REMARKS

Upon entry of the amendments, claims 13, 16, 32, and 34-46 will constitute the pending claims in the present application. Applicants have amended the language of claims 13, 36-37, and 39 for greater clarity. Applicants have added claim 45. Support for claim 45 may be found, for example, in paragraph 0071 of the published application (U.S. Application Publication No. 20040063627), which reads: "The term "DAPC" refers to "dystrophin-associated protein complex", a membrane complex which comprises dystrophin, α- and beta-dystroglycans, and the sarcoglycan transmembrane complex" (see FIG. 1), and in paragraph 193 which refers to a "mutated or absent DAPC component". Applicants have also added claim 46, which is supported (for instance) in Example 10. The amendments are fully supported by the specification, and contain no new matter.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

Information Disclosure Statement

The Examiner stated that she did not consider the IDS filed on October 30, 2007. Applicants take this opportunity to inform the Examiner of the related, co-pending applications (USSN 10/868,247 and 10/486,678) noted in the filing of October 30, 2007. Specifically, Applicants draw the Examiner's attention to the following office actions issued in the above-mentioned related applications:

1. Notice of Allowance dated September 27, 2007 in association with USSN 10/868,247;
2. Office Action dated April 26, 2007 in association with USSN 10/868,247;
3. Office Action dated November 15, 2006 in association with USSN 10/868,247;
4. Office Action dated December 18, 2006 in association with USSN 10/486,678;
5. Office Action dated June 13, 2006 in association with USSN 10/486,678.

Applicants will be happy to provide copies of any of the applications or office actions cited above upon request by the Examiner.

Claim Objections

The Examiner has objected to claim 36 as allegedly being improperly dependent on claim 32. The Examiner takes the position that claim 36, which recites "90% identical to amino acids 38-365 of SEQ ID NO: 9" is actually broader in scope than claim 32, which recites "90% identical to SEQ ID NO: 9". Applicants respectfully traverse.

Solely to expedite prosecution of the application, Applicants have amended claims 36-37 to correct claim dependency. Accordingly, reconsideration and withdrawal are respectfully requested.

Claim rejections under 35 U.S.C. §112, second paragraph

Claims 13, 16, 32, and 34-44 are rejected under 35 USC 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Office Action asserts that the term "an abnormal dystrophin-associated protein complex (DAPC)" in claim 13 is unclear. In addition, the Examiner argues that claim 39 omits essential steps. The Office Action then argues that claims 16, 32, 34-38, and 40-44 are indefinite for depending on claim 13 or 39. Applicants respectfully traverse for the reasons below.

Regarding the meaning of "abnormal DAPC" in claim 13, the definition would be clear to one of skill in the art based on the specification and the state of the art at the time of filing. Paragraph 0070 of the published application reads, "The term "abnormal" is used interchangeably herein with "aberrant" and refers to a molecule, or activity with differs from the wild type or normal molecule or activity. " Paragraph 0071 reads: "The term "DAPC" refers to "dystrophin-associated protein complex", a membrane complex, set forth in FIG. 1, which comprises dystrophin, α- and beta-dystroglycans, and the sarcoglycan transmembrane complex." In addition, the specification provides examples of abnormal DAPCs. For instance, paragraph 0172 summarizes DAPC abnormalities by referring to abnormalities in dystrophin, a DAPC component: "Dystrophin abnormalities are responsible for both the milder Becker's Muscular Dystrophy (BMD) and the severe Duchenne's Muscular Dystrophy (DMD). In BMD dystrophin is made, but it is abnormal in

either size and/or amount." Based on the explicit definitions and examples in the specification, one of skill in the art would instantly be able to construe the meaning of claim 13.

In addition to the information in the specification regarding "abnormal DAPC", the state of the art at the time of filing provides extensive guidance by which to interpret the term. Applicants concurrently submit three publications, Bonnemann *et al.*, Matsumura *et al.*, and Ozawa *et al.* (referred to as **Exhibits A, B, and C** respectively), which were published before the priority date of the instant application. These three articles show that characteristics of normal DAPC were known in the art. The publications also give examples of abnormal DAPC. For example, Exhibit A describes aspects of a normal DAPC on page 569 by stating that dystrophin "is a large subsarcolemmal rodlike molecule. Dystrophin's N-terminus is involved in binding cytoplasmic actin, whereas its C-terminus links to a large oligomeric complex of proteins that copurify with dystrophin". Exhibit A also states that the dystroglycan complex and sarcoglycan complex are some of these dystrophin-associated proteins (page 570). In addition, Exhibit B describes known DAPC abnormalities stating that "all of the DAPs were drastically reduced in the sarcolemma of mdx mice" (page 6) and on page 7 that "immunohistochemistry has shown reduced and/or patchy dystrophin staining along the sarcolemma, and immunoblot analysis has detected dystrophin of abnormal size and/or reduced quantity." Exhibit C adds that mdx mice "have a nonsense point mutation in the rod domain of the DMD gene and lack dystrophin" (page 1713), that "sarcoglycan complex expression is greatly reduced in DMD" (page 1714) and that "In BMD, the dystrophin rod is shorter that normal in most cases (resulting from a deletion of the DMD gene) or longer in others (resulting from duplication of the DMD gene)" (page 1714). Given that the identity of DAPC components were known, that structural information about their interactions was known, and that multiple examples of abnormal DAPC were known, one of skill in the art would have no difficulty construing the term "abnormal DAPC."

Turning now to claim 39, the Office Action argues that "it is not obvious what specific steps are intended by the claim to complete the claimed method." Applicants assert that additional steps are not necessary in this process claim because it is the specification, not the claims, that teaches one of skill in the art how to make and use / practice the claimed invention. One exemplary MuSK

activity assay is described in Example 10 in the specification. Applicants have added claim 46, which recites a specific MuSK activity assay.

The Office Action argues that claims 16, 32, 34-38, and 40-44 are indefinite for depending on claim 13 or 39. Applicants believe, based on the reasoning above, that claims 13 and 39 are definite. Claims depending therefrom are therefore definite as well.

In light of the remarks above, reconsideration and withdrawal of this rejection is respectfully requested.

Claim rejections under 35 U.S.C. §102(b)

Claims 13, 16, 32, and 34-44 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Ruoslahti *et al.*, US Patent No. 5,654,270. The Office Action states that Ruoslahti describes administering biglycan to wounded muscular tissue. The Examiner takes the position that the phrase "wherein the cell has an abnormal dystrophin-associated protein complex (DAPC)" encompasses cells in a wounded tissue. This broad reading of the term "abnormal DAPC" is based on the current indefiniteness rejection. Applicants respectfully traverse the rejection. Specifically, Applicants believe that the remarks in the previous section of the Response obviate the indefiniteness rejection. Accordingly, "wherein the cell has an abnormal dystrophin-associated protein complex (DAPC)" encompasses, for example, mutations in DAPC components, but excludes tissue that is merely wounded.

The Examiner has not stated why she believes that a wounded tissue has abnormal DAPC. To create a *prima facie* case of obviousness, the Examiner must show clear and convincing reasoning why a wound is expected to *inherently* have abnormal DAPC. It is established that "Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient." *Cont'l Can Co. USA, Inc. v. Monsanto Co.,* 948 F.2d 1264, 1269, 20 USPQ2d 1746, 1749 (Fed.Cir.1991) (emphasis in original). Indeed, it is difficult to imagine how a physical wound could cause a mutation in a DAPC component, or cause down-regulation of a DAPC component.

Since Ruoslahti does not teach administration of a biglycan therapeutic to a cell with abnormal DAPC, Applicants believe that withdrawal of the 102(b) rejection is appropriate. Applicants respectfully request reconsideration and withdrawal of the rejection.

Double Patenting Rejection

Claim 44 was rejected on the basis of alleged obviousness-type double patenting over claims 1-14 of U.S. Patent No. 6,864,236. Without conceding that the pending claims necessarily fully encompass the claims of the '236 patent, Applicants request that the Examiner hold the rejections made under the judicially created doctrine of obviousness-type double patenting in abeyance until otherwise allowable subject matter is identified in the instant application. Once allowable subject matter has been identified, Applicants will evaluate the filing of a terminal disclaimer or providing arguments in view of the claims pending at that time.

CONCLUSION

In view of the above remarks, Applicants believe the pending application is in condition for allowance.

Applicants believe no fee is due with this response other than those indicated on the accompanying transmittal. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. BURF-P02-006 from which the undersigned is authorized to draw.

Dated: 6/30/2008

Respectfully submitted,

By _____

Z. Angela Guo, Ph.D.
Registration No.: 54,144
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

Exhibit A

Beyond dystrophin: current progress in the muscular dystrophies

Carsten G. Bönnemann, MD*†, Elizabeth M. McNally, MD, PhD*, and Louis M. Kunkel, MD, PhD*

Major advances in the genetic understanding of the limb-girdle (LGMD) and congenital (CMD) muscular dystrophies have led to a new, genetically based classification of these disorders. The definition of the complex of dystrophin-associated proteins on a biochemical and subsequently genetic level has greatly accelerated this progress by providing candidate genes to complement or replace the process of linkage analysis either in families with muscular dystrophy or in sporadic cases. The major components of the dystrophin-associated proteins now known to be involved in muscular dystrophy besides dystrophin itself are the sarcoglycan complex and the α2-chain (merosin) of laminin-2 in the extracellular matrix. Mutations in the various sarcoglycans account for four types of autosomal recessive LGMD of varying severity (types 2C through 2F), including severe childhood-onset presentations. One type of autosomal recessive LGMD (type 2A) is caused by mutations in the protease calpain-3, whereas the gene for type 2B has not yet been identified, although the responsible locus has been assigned to chromosome 2p13. There are different autosomal dominant forms as well, one of which has been mapped to chromosome 5q31. With regard to CMDs, the major breakthrough involves a type of "classic" CMD with abnormalities of the white matter on magnetic resonance imaging of the brain. These patients show deficiencies of the laminin α2-chain, and mutations in the corresponding gene have been identified. The group of laminin α2-chain–positive classic CMD likely is heterogeneous. Among the group of CMDs with abnormalities of brain formation and mental retardation, genetic, immunohistochemical, and clinical differences are now beginning to emerge to help in the distinction between Fukuyama muscular dystrophy, the Walker-Warburg syndrome, and muscle-eye-brain disease.

*Division of Genetics and Howard Hughes Medical Institute, and †Department of Neurology, The Children's Hospital, 300 Longwood Avenue, Boston, MA 02115, USA.

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Abbreviations

BMD	Becker muscular dystrophy
CK	creatine kinase
CMD	congenital muscular dystrophy
CNS	central nervous system
DMD	Duchenne muscular dystrophy
LGMD	limb-girdle muscular dystrophy
MRI	magnetic resonance imaging
SCARMD	severe childhood autosomal recessive muscular dystrophy

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Progress in the genetic definition of muscular dystrophies has been driven mainly by two major approaches. One approach is purely genetic, starting with the establishment of genetic linkage to anonymous DNA polymorphisms in the human genome. The identification of candidate genes for muscular dystrophy is what constitutes the second major approach, which complements the first. Here the main focus has been on the biochemical, and subsequently genetic, characterization of proteins associating and interacting with dystrophin, the first protein found to be mutated in a human muscular dystrophy. This field of study started with the discovery that dystrophin was associated with a complex of glycoproteins anchoring it to the sarcolemma [1–3], immediately raising questions about the potential involvement of these glycoproteins in muscular dystrophies.

Muscular dystrophies are typically progressive disorders mainly of striated muscle that lead to breakdown of muscle integrity. The histologic picture shows variation in fiber size, muscle cell necrosis and regeneration, and often proliferation of connective and adipose tissue. Although there is a wide variety of clinical types and variations, several larger groups of muscular dystrophies can be identified (Table 1). This review concentrates on the autosomal limb-girdle muscular dystrophies (LGMDs) and the congenital muscular dystrophies (CMDs), because the entities in these two broad groups both exemplify the importance of the dystrophin-associated proteins in the development of muscular dystrophy and underline the importance of careful genetic analysis of families that have what looks like a fairly homogeneous clinical spectrum. Duchenne (DMD) and Becker (BMD) muscular dystrophies, although clearly related to the disorders discussed here, will not be addressed in this update. Because this review often draws rather extensively on the concept of dystrophin-associated proteins, we briefly introduce the current concepts about the biochemical organization of this complex of proteins.

The complex of dystrophin-associated proteins

Dystrophin, the protein product of the gene mutated in DMD and BMD, is a large subsarcolemmal rodlike molecule [4]. Dystrophin's N-terminus is involved in binding cytoplasmic actin, whereas its C-terminus links to a large oligomeric complex of proteins that copurify with dystrophin in wheat-germ agglutinin-affinity purified

Table 1

The major muscular dystrophies*

Congenital muscular dystrophies (see Table 2)
Classic or pure congenital muscular dystrophies
Congenital muscular dystrophies with structural central nervous system anomalies and mental retardation
Limb-girdle muscular dystrophies (see Table 2)
Duchenne and Becker muscular dystrophies (XR, Xp21)
Autosomal dominant forms
Autosomal recessive forms
Distal muscular dystrophies
Miyoshi (AR, 2p21, may be identical with limb-girdle muscular dystrophy 2B)
Welander (AD)
Nonaka (AR)
Tibial (Udd) (AD)
Bethlem myopathy (AD, 21q22.3, 2q37)
Emery-Dreifuss muscular dystrophy (XR, Xq28 plus autosomal locus/loci)
Facioscapulohumeral dystrophy (AD, 4q35, plus other autosomal locus/loci)
Oculopharyngeal muscular dystrophy (AD, 14q11.2-q13, plus other locus/loci)
Myotonic dystrophy (AD, 19q13.3)

*This table is not meant to be comprehensive. Rather it provides context for the disorders discussed in this review. Mode of inheritance and genetic location, when known, are given in parentheses. AD–autosomal dominant; AR–autosomal recessive; XR–X-linked recessive.

skeletal muscle membrane preparations (Fig. 1) [2,3,5]. (For a recent review on dystrophin and its domains and isoforms, see Sadoulet-Puccio and Kunkel [6•]). Several subgroups among these dystrophin-associated proteins can be differentiated (Fig. 1). The first of these is the dystroglycan complex [7]. β-Dystroglycan (43 kD molecular weight) is a transmembrane protein that binds to the C-terminal cysteine-rich region of dystrophin within the cell and to α-dystroglycan (156 kD) at the extracellular site. α-Dystroglycan itself binds to the extracellular matrix component laminin-2 (more precisely the laminin α2-chain of this heterotrimer) [8], thus completing a link from the cytoskeleton to the extracellular matrix. The second group has been named the sarcoglycan complex, after it was shown that its members could be separated from the other proteins by using special detergent conditions [9]. This complex is composed of α-sarcoglycan (50 kD molecular weight, formerly known as adhalin), β-sarcoglycan (43 kD), γ-sarcoglycan (35 kD), and likely also the newly indentified δ-sarcoglycan (35 kD) [10•,11•]. The biological function of this complex is currently unknown. However, its pivotal role in maintaining muscle cell integrity is emphasized by the fact that mutations in any one of the known sarcoglycans can cause muscular dystrophy [12••]. A hydrophobic protein of 25 kD also copurifies with dystrophin and may be identical with muscle caveolin [13], although this component is probably heterogeneous. A number of intracellular proteins constitute a third group, which contains the syntrophins (59 kD) that directly bind to the C-terminus of dystrophin. Also intracellular is dystrobrevin, a dystrophin relative as well as a dystrophin-associated protein that also has a subsarcolemmal localization and links to dystrophin and to the syntrophins [14]. (For a recent review about the dystrophin-associated proteins, see Tinsley *et al.* [15] and Ozawa *et al.* [16•]).

As mentioned earlier, the dystroglycan complex connects to laminin-2, a heterotrimer of the extracellular space. Laminin-2 is fairly specific for skeletal muscle, cardiac muscle, and peripheral nerve but can also be found in the brain. It is composed of a laminin α2-chain (formerly known as merosin, or M-chain), complexed with β1- and γ1-chains. In addition to providing a structural link to other components of the basal membrane, laminins also play a role in cell adhesion and possibly cell migration, as well as axon guidance and outgrowth [17].

Limb-girdle muscular dystrophies

A profound redefinition of classification systems has taken place in the LGMDs as a result of advances in the genetic and biochemical understanding of these disorders (Table 2). The diagnostic criteria for LGMDs have been summarized by Bushby [18] for a European Neuromuscular Centre–sponsored working group on LGMDs. She provides both inclusion and exclusion criteria to separate this group of disorders clinically from other neuromuscular conditions that present with weakness in a limb-girdle distribution. The proposed criteria consist of predominantly proximal weakness affecting the pelvic and shoulder girdles as well as weakness of the muscles of the trunk, with more distal weakness mostly not occurring until later in the disease progression. The facial muscles are usually spared or only minimally involved, and extraocular muscles are completely spared. Creatine kinase (CK) activity in the serum can be normal or mildly to grossly elevated, electrophysiologic studies are myopathic, and muscle biopsy reveals myopathic to frankly dystrophic features. The exclusion of other conditions such as disorders of the lower motor neuron and peripheral nerve, as well as inflammatory, metabolic, mitochondrial, structural, or congenital myopathies, is essential.

Clinically this group of disorders is uniformly progressive, although there is remarkable variability with respect to age of onset and rate of progression within the group as a whole as well as within a single genetically defined entity. DMD and BMD would certainly conform with these criteria and indeed could be classified as X-linked LGMD, although for historical reasons this terminology has not been adopted.

The autosomal LGMDs may be classified in two complementary ways: by mode of inheritance and by status of the sarcoglycan complex. The new nomenclature for LGMDs outlined by Bushby and Beckmann [19•] follows the pattern of inheritance. The autosomal dominant forms are designated as LGMD type 1 and the autosomal recessive forms as type 2, both with additional lettering following the chronology of the genetic definition (*ie*, establish-

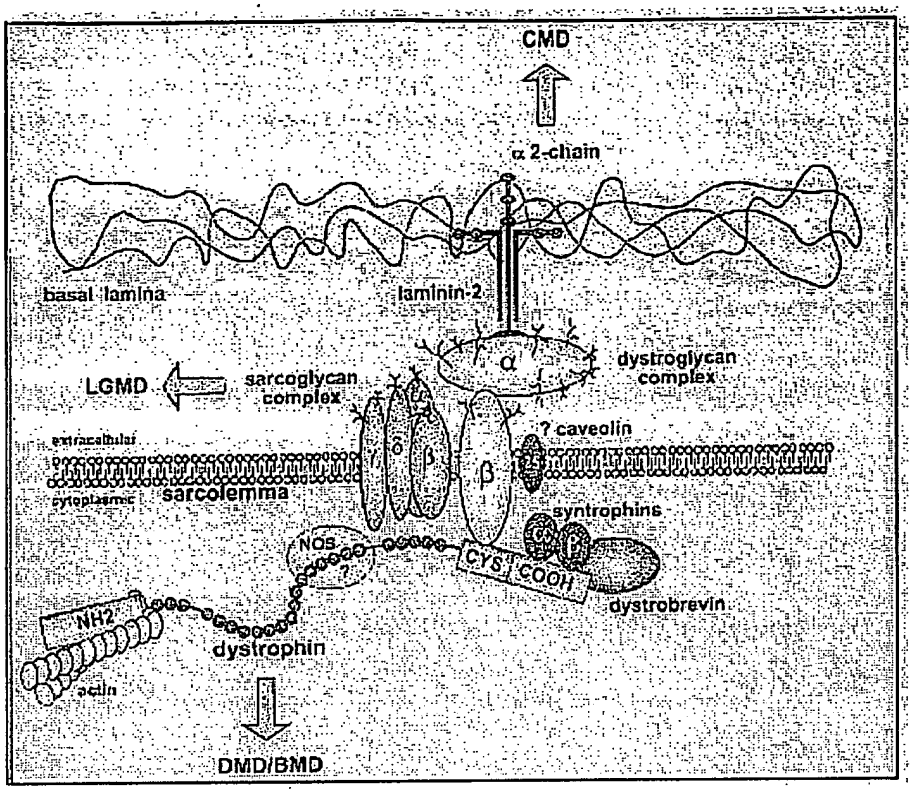

Fig. 1. The dystrophin-associated protein complex. Given the active and changing nature of the field, this diagram reflects assumptions at the time of writing and may very well change appearance as new data accumulate. Neuronal nitric oxide synthetase (NOS) also purifies with the dystrophin complex [125•], but the exact interactions of NOS within the complex are not entirely clear. See text for more detailed discussion of the other various components. *Arrows* indicate the components mutated in the various muscular dystrophies associated with abnormalities in the complex. BMD–Becker muscular dystrophy; CMD–congenital muscular dystrophy; DMD–Duchenne muscular dystrophy; LGMD–limb-girdle muscular dystrophy.

ment of a genetic map position) of the particular subtype. We will follow this proposed nomenclature. In this system, DMD and BMD could conceivably be designated as LGMD X.

Autosomal recessive limb-girdle muscular dystrophies

In general, the recessive LGMDs tend to have an earlier onset and follow a more severe clinical course than the autosomal dominant forms. However, there is substantial variability of clinical severity within the group as a whole as well as within several of its separate entities. It has become clear, for example, that severe childhood autosomal recessive muscular dystrophy (SCARMD) and DMD are not diseases separate in principle from milder manifestations, but that both degrees of severity can occur as a spectrum within the same genetic entity. This is a situation not unlike the realization that DMD and BMD were caused by mutations within the same gene. It has been estimated that even before dystrophin studies are done, about 8% to 12% of "sporadic" (*ie*, without a family history) male patients with LGMD will have a recessive LGMD [20,21].

Limb-girdle muscular dystrophy 2A

Limb-girdle muscular dystrophy 2A was the first autosomal LGMD to be put on the genetic map. Extensively studied in a small community in Réunion Island off Madagascar [22•], the onset is usually at around 10 years of age, followed by a slowly progressive deterioration leading to loss of ambulation at about 20 years of age. Earlier onset of symptoms correlates with more rapid disease progression and vice versa. Linkage of LGMD 2A to chromosome 15q was first established in this community [23] and subsequently confirmed and extended in Brazilian [24] and in North American Indiana Amish [25] families. The genetic region was subsequently narrowed [25], and a cDNA corresponding to the gene for the muscle-specific neutral calcium–dependent protease calpain-3 was identified in the critical interval [26].

Mutations in this gene were detected in affected individuals and found to segregate with the disease [27••]. The mutations included nonsense (associated with a more severe phenotype) and missense mutations. How mutations in this protease cause muscular dystrophy is an open question at present, although they appear to implicate dysregulation of (regulatory) proteolysis in the muscle cell.

Elucidating the physiologic role of calpain-3 will be extremely important, and the recent finding of an association of calpain-3 with connectin (titin) in the muscle cell is an important step into that direction [28•]. Dystrophin and the sarcoglycan complex are mostly normal by immunohistochemical analysis in this disorder [29•]. Although the genetic isolation of the Réunion community and genealogic evidence for inbreeding within the community would seem to favor the segregation of a

Table 2

Classification, genetic loci, gene products, and immunohistochemistry*

Designation	Genetic locus	Protein product	Immunohistochemistry for		
			Dystrophin	Sarcoglycan	Laminin-α2 chain
LGMDs					
X-chromosomal recessive					
Duchenne and Becker muscular dystropies	Xp21	Dystrophin	neg to ↓↓↓	↓ to ↓↓	nl
Autosomal dominant					
LGMD 1A	5q31	–	nl	nl	nl
LGMD 1B	1q11–21	–	nl	nl	nl
LGMD 1C	–	–			
Autosomal recessive			nl	nl	nl
LGMD 2A	15q15	Calpain 3	nl	nl	nl
LGMD 2B	2p13	–	nl to ↓	neg to var ↓↓↓	nl
LGMD 2C	13q12	γ-Sarcoglycan	nl to (↓)	neg to var ↓↓↓	nl
LGMD 2D	17q21	α-Sarcoglycan	nl to ↓	neg to var ↓↓↓	nl
LGMD 2E	4q12	β-Sarcoglycan	nl to ↓	neg to var ↓↓↓	nl
LGMD 2F	5q33-34	δ-Sarcoglycan			
LGMD 2G	–	–			
Congenital muscular dystrophies					
Classic CMDs			nl	nl	nl
Laminin α2-chain–positive	–	–	nl	nl	neg to ↓↓↓
Laminin α2-chain–deficient	6q22-23	Laminin α2-chain			
CMD with central nervous system abnormalities			nl	nl	↓ to ↓↓
Fukuyama muscular dystrophies	9q31-33	–	nl	– (↓ for α-sarcoglycan)	nl
Walker-Warburg syndrome	–	–	nl	nl	↓
Muscle-eye-brain disease	–	–			

*The indications about immunohistochemical changes should serve as orientation only and can vary from case to case.
CMD—congenital muscular dystrophy; LGMD—limb-girdle muscular dystrophy; neg—negative; nl—normal; var—variable.

single mutation arising from a founder effect, a number of distinct mutations and haplotypes were seen. This phenomenon has yet to be explained fully. A digenic model of inheritance, that is, the existence of a second, modifying gene locus, has been postulated [27••,30]. In the Northern Indiana Amish families, a single mutation following classic autosomal recessive inheritance has been identified [31].

Limb-girdle muscular dystrophy 2B

In this autosomal recessive LGMD, genetic linkage has been established to chromosome 2p13 in Palestinian and Italian families [32] as well as in Brazilian pedigrees [33]. Within the group of autosomal recessive LGMDs, the phenotype is on the mild end of the spectrum, with age of onset usually after 15 years and relatively slow progression, although exceptions with more rapid deterioration do exist and marked intrafamilial variability has also been seen [32–34,35•]. The CK levels are usually extremely elevated (in the ten thousands). The effort to identify the responsible gene currently has narrowed down the critical region to 400 to 600 kb [36,37]. In this type of LGMD, similar to type 2A, dystrophin and the sarcoglycan complex are normal by immunohistochemistry [29•] (K. Bushby, Personal communication).

A clinically quite different appearing disorder, Miyoshi myopathy, usually is classified as a distal muscular dystrophy, in which symptoms start distally in the legs with the inability to stand on toes because of weakness in the gastrocnemius muscle. This recessive dystrophy has recently been mapped to the same region on chromosome 2 where LGMD 2B resides [38•]. It is notable that later in Miyoshi myopathy, there is involvement of proximal muscles also. In LGMD 2B, on the other hand, there is evidence for early involvement of the gastrocnemius muscle, especially via CT of the muscle [35•]. Therefore, there appears to be the distinct possibility that these two phenotypically different myopathies are allelic (*ie*, caused by different mutations within the same gene). The variabilty discussed in the LGMDs usually reflects differences in the age of onset and the rapidity of progression, but not in the distribution of the weakness, so that such a finding would add an additional dimension to the genetics of LGMDs.

Sarcoglycan-deficient autosomal recessive limb-girdle muscular dystrophies

Four autosomal recessive LGMDs (types 2C through 2F) are caused by mutations in members of the sarcoglycan complex. An intriguing finding that emerged from the analysis of these disorders is the apparent interdependent nature of the sarcoglycan proteins. Even before the first mutations in the sarcoglycans were described, it had been observed that there were patients with LGMD who showed a selective absence of the sarcoglycan complex by

immunohistochemistry on muscle [39]. It is now clear that mutations in any one of the four known members lead to more or less pronounced secondary deficiencies of the other components of the complex, indicating the importance of the integrity of the entire complex for the prevention of muscle cell degeneration.

As mentioned earlier, the exact biological function of the sarcoglycan complex is not yet known. There are certain structural similarities between the four known members of the complex that may also indicate that these molecules are related functionally. All have a single transmembrane domain and extracellular glycosylation consensus sites as well as intracellular consensus sites for serine or threonine phosphorylation in three. All four have clusters of extracellular cysteine residues, which form a peculiar partial epidermal growth factor–like module in β-, γ- and δ-sarcoglycan. There is some biochemical evidence for actual physical interaction between the components of the complex [9,40], but it is not known how this is mediated. α-, γ-, and δ-sarcoglycan are expressed exclusively in muscle, whereas β-sarcoglycan has a more widespread expression, including the brain and kidneys [41,42••].

Certain clinical features appear to be common to the group of autosomal recessive sarcoglycan-deficient LGMDs: taken as a group, the clinical involvement and progression tend to be more severe than in both the autosomal dominant and the sarcoglycan-positive autosomal recessive types 2A and 2B LGMDs. However, as will be discussed in the following section, there is marked clinical variability within this group and within its separate genetic entities. Early in the course of the disease, there are often dramatic increases in CK values, although these can decline considerably with disease progression. In addition, calf hypertrophy is often prominent in the phases of early disease progression but less pronounced after loss of ambulation. Mental development appears normal and no higher incidence of mental retardation has become apparent (in contrast to DMD), although the number of identified patients is still small.

Cardiac involvement does not appear to be clinically evident in most cases but clearly requires close attention. Fadic *et al.* [43] described a patient with prominent cardiomyopathy leading to cardiac failure and cardiac transplantation in whom α-sarcoglycan was found to be deficient by immunohistochemistry in heart and skeletal muscle, but a primary mutation in a sarcoglycan gene was not reported. For addressing this problem, an important animal model is provided by the cardiomyopathic and dystrophic hamster BIO 14.6, which is completely deficient for sarcoglycan in heart and skeletal muscle by immunohistochemistry [44]. Mutations in α-sarcoglycan [45] and in β- and γ-sarcoglycan [46] have been excluded in this hamster, but δ-sarcoglycan has not yet been ruled out.

Limb-girdle muscular dystrophy 2C

In 1983, Ben Hamida *et al.* [47] described an autosomal recessive muscular dystrophy prevalent in Tunisia resembling DMD in severity. Although this disorder has been referred to as SCARMD, this is somewhat of a misnomer because there can be marked variability in clinical severity even within the same family, with some patients showing later onset or slower progression. Abnormalities of immunostaining for the 50-kD α-sarcoglycan were shown in some patients [48], suggesting an alteration of the sarcoglycan complex in this disorder.

Linkage to chromosome 13q12 [49] was established in Tunisian, Algerian [50], and other northern African families [51]. Recently, the γ-sarcoglycan cDNA was cloned and mapped to this critical region on chromosome 13 [52••]. In all 13q12-linked Tunisian families tested the same deletion of a single thymidine was found [52••]. This homogeneity had been expected given the existence of strong linkage disequilibrium within this population, a reflection of the degree of inbreeding. The mutation causes a frameshift leading to a premature translational termination codon and predicts a truncated protein. Meanwhile, it has become clear that γ-sarcoglycan mutations occur worldwide [52••,53,54]. Interestingly, in Brazilian families carrying the same deletion on the same haplotype that was described in the North African patients, marked variation in severity was seen between families, suggesting the existence of a modifying locus [54]. Jung *et al.* [40] have subsequently also shown the absence of γ-sarcoglycan protein with severely reduced α- and β-sarcoglycan by Western blot analysis in North African patients.

Limb-girdle muscular dystrophy 2D

Missense mutations in α-sarcoglycan (formerly known as adhalin) were the first demonstrated mutations in a dystrophin-associated protein causing muscular dystrophy. These mutations were shown in a family with genetic linkage of the disease to the α-sarcoglycan gene on chromosome 17 [55]. Subsequently, mutations in α-sarcoglycan have been reported from a number of different groups [56•,57–60]. Again there is wide variability in the clinical severity, with a tendency for a larger number of milder cases, when compared with the other sarcoglycan disorders. In α-sarcoglycan there is a trend for missense mutations to cause a relatively milder phenotype when compared with nonsense and truncating mutations [56•,58–60], although exceptions to this rule do occur, as in one of the cases reported by Kawai *et al.* [57]. The missense mutation Arg77Cys is the single most frequent mutation in this group [55,56•,59], independent of ethnic background. LGMD 2D may be the most common of the known sarcoglycan-deficient LGMDs [61], although the experience of more centers needs to be pooled to allow for such an assessment. A potential ascer-

tainment bias may exist as a result of the use of the commercially available anti–α-sarcoglycan antibody as the screening tool.

Limb-girdle muscular dystrophy 2E

Mutations in the β-sarcoglycan gene were discovered by two independent approaches. Cloning of the β-sarcoglycan cDNA allowed mapping of the gene to chromosome 4q12 [41,42••], a location to which no muscular dystrophy had yet been mapped. In one approach, screening of patients with unexplained muscular dystrophy and normal dystrophin resulted in the identification of a young girl with the onset of limb-girdle weakness at 3 years of age who had truncating mutations on both of her β-sarcoglycan gene alleles [41]. The second approach was based on the discovery by linkage analysis that in the Indiana Amish community there was a second genomic locus, in addition to LGMD 2A, responsible for muscular dystrophy [62]. Linkage to chromosome 4q12 was established and a homozygous missense mutation identified in β-sarcoglycan, causing a phenotype of intermediate but variable severity [42••].

More mutations have now been identified in different populations [61,63]. In addition to the truncating mutations causing a severe phenotype with early onset and a DMD-like progression, there appears to be a higher proportion of missense mutations in β-sarcoglycan causing an equally severe phenotype. Although β-sarcoglycan is the only sarcoglycan that is expressed in tissues outside of muscle, including the brain and kidney [41,42••], there is no indication for dysfunction of extramuscular organs in the patients indentified so far. It should be noted that the expression of the other sarcoglycan proteins is restricted to muscle, so that the sarcoglycan complex as such only exists in muscle.

Limb-girdle muscular dystrophy 2F

This is the latest addition to the family of sarcoglycan-deficient muscular dystrophies. In Brazil, several large families with autosomal recessive LGMD had been very carefully analyzed genetically with all the hitherto known genetic markers for the different types of LGMD. In the course of this analysis a new genetic location on chromosome 5q33–34 was identified in two families with a severe phenotype and total sarcoglycan deficiency on the muscle biopsy [64•]. Simultaneously, Nigro *et al.* [10•] identified a new member of the sarcoglycan protein family. By database homology searches with the γ-sarcoglycan sequence, these workers discovered a partial expressed sequence tag corresponding to a novel 35-kD protein, δ-sarcoglycan, mapping to the same region on chromosome 5 as the two families from Brazil. A homozygous missense mutation was discovered in δ-sarcoglycan in the Brazilian families [65••], confirming their status as a sarcoglycan disorder. The existence of δ-sarcoglycan was confirmed subsequently by a second group [11•].

Other autosomal recessive limb-girdle muscular dystrophies

By excluding all known genetic locations for autosomal recessive LGMD, Passos-Bueno *et al.* [64•] have shown that there is at least one more locus for autosomal recessive LGMD—a sarcoglycan-positive type with a severe phenotype. Preliminary screening experience of α-sarcoglycan–deficient muscle biopsies for mutations in α-, β-, or γ-sarcoglycan, but not δ-sarcoglycan, is available [61]. In Hoffmann *et al.*'s [61] study, only 52% in their α-sarcoglycan–deficient sample could be accounted for by α-, β-, or γ-sarcoglycan gene mutations. Unless the remaining cases are accounted for by δ-sarcoglycan mutations, this could indicate that there may be other genes causing sarcoglycan-deficient LGMD.

Autosomal dominant limb-girdle muscular dystrophies

Limb-girdle muscular dystrophy 1A

Limb-girdle muscular dystrophy 1A was genetically defined by linkage analysis [66,67] to a 7-cM interval on chromosome 5q31 in a large family with LGMD [68]. Clinically, the onset of symptoms is in young adulthood, and the disease progression is slow, with loss of ambulation only very late in the course of the disease in some members of the family. A curious and potentially specific finding of a subgroup of affected members in this family was dysarthria probably resulting from palatal weakness, which occurred even before the onset of frank limb-girdle weakness. The known dystrophin-associated proteins are normal by immunohistochemistry in this disorder (Bönnemann *et al.*, Unpublished observations). The responsible gene has not yet been isolated, although a positional cloning effort is under way.

Other autosomal dominant limb-girdle muscular dystrophies

The existence of additional loci for autosomal dominant LGMD has become clear. Speer *et al.* [69] demonstrated that three additional families with autosomal dominant LGMD were not linked genetically to the LGMD 1A locus on chromosome 5q31. These families did not have dysarthria or prominent cardiac symptoms. Several families with a combination of prominent cardiomyopathy and LGMD had been reported over the years. More recently, three families with a distinct combination of LGMD followed by a cardiomyopathy presenting with dysrhythmias and atrioventricular conduction block as well as dilated cardiomyopathy later in the course were reported as a clinically homogeneous group [70]. This autosomal dominant LGMD with cardiomyopathy has now been genetically assigned to chromosome 1q11–21 and should be designated as LGMD 1B (van der Kooi *et al.*, Unpublished data.)

Bethlem myopathy is an autosomal dominant proximal myopathy of early onset, very slow progression, and early occurrence of multiple contractures. Clinically it is thus related to the LGMDs but has a characteristic phenotype. This condition has recently been mapped to chromo-

somes 21q22.3 [71] and 2q37 [72], to which regions different subunit genes of type VI collagen map. Mutations in two of the subunits (COL6A1 and COL6A2) were just confirmed for the 21q22.3 locus [73••]. These findings also extend the pathophysiologic chain of events now into the connective tissue around the muscle cell.

Evaluation and diagnosis of limb-girdle muscular dystrophies

The evaluation of patients who conform to the criteria for an LGMD must take into account the great genetic heterogeneity underlying the phenotype. Arriving at a specific genetic diagnosis may not change the management of the affected individual short of raising hopes for some form of gene therapy in the future, but it does provide a sound basis for genetic counseling as well as the prospect of a prenatal diagnosis in selected families. There are currently very few purely clinical clues to guide in the diagnosis. As outlined earlier, the degree of severity and the age of onset can provide rough guides as to whether one is dealing with a dominant or recessive form when confronted with a sporadic patient. However, the broad clinical variability, especially of the recessive variants, must be taken into account. Among the recessive group, there now is evidence for a more characteristic pattern of muscle involvement in LGMD 2A [22•] and 2B [35•], the recognition of which may be aided by muscle imaging, especially early in the disease. CK levels may be helpful in the active state of the disease, when normal values make a disorder of the sarcoglycan complex less likely, but can be deceptively low later in the course. The same can be said about calf hypertrophy. When the family history is informative, the mode of inheritance may become clear, allowing differentiation between autosomal dominant, recessive, or X-linked forms.

Analysis of a muscle biopsy specimen is crucial in every patient, except for males with clear dystrophin gene mutations on DNA analysis. (Dystrophin DNA analysis for deletions should probably be done in every male patient with LGMD.) Given the grave implications of a diagnosis of DMD, some clinicians prefer to perform a muscle biopsy even when the DNA analysis is positive for a deletion in order to confirm the diagnosis by an independent means. The biopsy should be examined by standard light microscopy for change compatible with muscular dystrophy and then by immunohistochemistry with antibodies against dystrophin and the various sarcoglycans. For the following statements one should bear in mind that immunofluorescence analysis of muscle biopsy sections is not quantitative but allows for only rough impressions about amounts of immunoreactivity. Antibodies are only commercially available at the moment for α- and γ-sarcoglycan.

In the case of biopsies in which there is total absence of dystrophin on immunohistochemistry (and Western blot analysis), a diagnosis of DMD can be made. However, in cases in which there appears to be diminished dystrophin staining, the differential diagnosis must include both BMD and disorders of the sarcoglycan complex, because secondary reduction of dystrophin on immunohistochemistry has been seen in a number of patients with primary sarcoglycan disorders [29•]. Therefore, dystrophin analysis by Western blotting should be done in all cases with abnormalities of dystrophin immunofluorescence. A dystrophin molecule of abnormal molecular mass confirms a diagnosis of BMD. However, a dystrophin molecule of normal molecular mass but severely reduced quantity could still be compatible with the diagnosis of BMD, because mutations in the promoter of the dystrophin gene affecting the quantity of dystrophin produced or missense mutations affecting the stability of the protein are conceivable.

If dystrophin and the sarcoglycans are present at the sarcolemma in normal amounts, the disorder in all likelihood does not involve the sarcoglycan genes. In informative families, inclusion or exclusion of the known genetic loci can be attempted or direct mutation analysis in the calpain-3 gene can be undertaken for LGMD 2A. Calpain-3 antibodies are not yet available, so it is not known whether calpain immunostaining is abnormal in these patients.

If the sarcoglycans are deficient, relative intensities of immunolabelling may be significant [29•]. In cases in which one of the sarcoglycans is completely absent while the other components show diminished staining, the subsequent genetic analysis for mutations in the different sarcoglycan genes might be predicated on the assumption that the completely absent component points to the mutated gene. Such an approach would be significantly reinforced by Western blot analysis for the sarcoglycans [74].

Most of the screening for disorders has been done through the use of the anti–α-sarcoglycan antibody [60,61,75,76], so that most experience has accumulated with this antibody. However, it may be necessary to use at least both anti-α- and anti–γ-sarcoglycan antibodies in the immunohistochemical analysis, because γ-sarcoglycan mutations may cause only mild reductions in α-sarcoglycan immunostaining but with a much clearer reduction in γ-sarcoglycan [29•]. More often, however, the pattern obtained by immunohistochemistry is either a complete absence of the entire complex or diffusely diminished staining for all the components and is not helpful. In these cases mutation analysis for all sarcoglycan genes must be performed in order to arrive at a specific diagnosis. It seems, however, that especially in cases with partial deficiencies a mutation in one of the sarcoglycan genes may often not be ascertainable [61]. Genetic analysis for mutations currently must be performed by specialized laboratories involved in research on these disorders.

Congenital muscular dystrophies

Congenital muscular dystrophies are usually clinically apparent at birth or in the first few months of life with hypotonia, weakness, and variable degrees of arthrogryposis multiplex congenita in some cases. CK is often elevated but can be normal. The muscle biopsy shows changes consistent with a muscular dystrophy, frequently with impressive connective tissue proliferation.

In general, two broad groups can be distinguished by virtue of the presence or absence of abnormalities of brain formation evident on neuroimaging studies or on autopsy examination of the brain (Table 2). The first group, children with what appears to be "pure" CMD without clinically apparent brain dysfunction or malformations of the brain, has been referred to as "classical" CMD. This group is clearly heterogeneous. The most important subclassifying feature here is the presence or absence of the laminin α2-chain (merosin) by immunohistochemistry on the muscle biopsy. In the second group, affected individuals have varying degrees of mental retardation, evidence of diverse malformations of the brain, and often clinical involvement of the eyes as well. This group includes Fukuyama muscular dystrophy, Walker-Warburg syndrome, and muscle-eye-brain disease. As will be seen in the following sections, these distinctions are not entirely precise in all cases and should probably not be overemphasized.

Laminin α2-chain–deficient classic congenital muscular dystrophy

It has become apparent through the application of neuroimaging techniques that there are patients with classic CMD who have evidence for an abnormality of the white matter resembling leukodystrophy on neuroimaging studies. These neuroimaging findings were concordant among siblings in a given family with CMD [77]. Independently, Tomé *et al.* [78] recognized that by immunohistochemistry a significant number of patients with classic CMD had a deficiency of the α2-chain on muscle biopsy. A concomitant increased staining for the α1-chain, a component of laminin-1, can be observed in these cases [78,79]. It became evident that the laminin α2-chain deficiency correlated with the abnormal findings of the white matter on T_2-weighted cranial magnetic resonance imaging (MRI) studies, thus defining a distinct subgroup among the patients with CMD.

Linkage analysis by homozygosity mapping in consanguineous pedigrees with laminin α2-chain–deficient CMD mapped the gene to chromosome 6q22–23 [80], the same region where the human laminin α2-chain gene, *LAMA2*, resides [81]. Subsequently, the first mutations in the human gene were reported in two families [82••]. They were splice-site and nonsense mutations and were predicted to cause truncations of the protein. More recently, a missense mutation in a conserved EGF-like module of the protein was reported as well [83], showing a diminished, but not absent, pattern of staining for the laminin α2-chain on the muscle biopsy.

By subdividing the group of classic CMDs into those that are laminin α2-chain–deficient versus those that are laminin α2-chain–positive, clinical differences between the two have become apparent [84•,85–88]. In the laminin α2-chain–deficient patients, the initial neonatal presentation was more severe, with a higher percentage of infants presenting with arthrogryposis, and these patients also have a far worse prognosis for ambulation. In fact, none of the α2-chain–deficient children achieved independent ambulation, whereas almost all of the positive patients eventually did, albeit sometimes with assisting devices. In addition, serum CK values on the whole appear to be higher in the laminin α2-chain–deficient group, usually around or above 1000.

All α2-chain–deficient patients studied with cranial MRI also had abnormalities of the white matter on T_2-weighted images that were not seen in the α2-chain–positive group. Careful electrophysiologic studies can further define the clinical phenotype. As might be expected from the expression of the laminin α2-chain in peripheral nerve and brain, slowing of motor nerve conduction velocities [89] as well as delayed somatosensory evoked potentials [90] were found, whereas abnormalities of visual evoked responses were less consistent.

These findings of central nervous system (CNS) and peripheral nervous system involvement become more pronounced with age and may be only minimal early in the course of the disease [91]. On standard neurologic assessment there is no indication for more widespread neurologic dysfunction in the children with α2-laminin–negative CMD. This may seem surprising, given the extent of the white matter abnormalities seen on MRI. A recent study suggests that there may be deficiencies in a number of semiquantitative motor coordination tasks as well as visuomotor processing tasks [92]. There now is evidence that the phenotype of laminin α2-chain–deficient CMD is broadening. On the one hand are patients with very mild disease who function well into adulthood (F. Tomé, personal communication), whereas on the other hand, three patients have now been identified with laminin α2-chain–deficient CMD who also had evidence of focal cortical dysgenesis on brain imaging [93]. Therefore, CMDs can no longer be clearly subdivided based on the presence or absence of abnormalities of brain formation. Additional evidence for the broadening phenotype of laminin α2-chain–deficient classic CMD has been provided by Herrmann *et al.* [94], describing an intermediate phenotype with incomplete deficiency, later onset, and achievement of ambulation. A comprehensive summary of the latest European Neuromuscular Centre–sponsored workshop on congeni-

tal muscular dystrophy with a discussion of the most recent advances has just been published [95]. These authors also point out the occurrence of seizures in laminin α2-chain–deficient CMD even without evidence of cortical dysplasia.

An animal model is provided by the mouse mutants *dy/dy* and *dy²ʲ/dy²ʲ*. These mice are also laminin α2-chain–deficient by immunohistochemistry [96–98] and carry mutations in the murine gene for the laminin α2-chain [99,100].

Investigations into the pathophysiology of laminin α2-chain deficiency and its relation to the development of muscular dystrophy as well as the apparent CNS dysmyelination are only now beginning. No autopsy studies of patients with proven α2-laminin–negative CMD have been published yet. In both the laminin α2-chain–deficient CMD and the *dy/dy* mouse, abnormalities of the basal lamina can be demonstrated ultrastructurally [98,101,102]. In the normal human CNS, the laminin α2-chain appears to be expressed in the blood-brain barrier but not in oligodendrocytes [103]. It will also be important as a short-term goal to investigate whether all laminin α2-chain–deficient patients do carry mutations in the *LAMA2* gene or whether there will be genetic heterogeneity similar to the sarcoglycan-negative LGMDs. Prenatal diagnosis may become possible by direct gene analysis in selected families. The laminin α2-chain is expressed in chorionic villus samples from human placenta [104], so that chorionic villus biopsy may become a feasible alternative way for prenatal diagnosis in this mostly severe disorder.

Laminin α2-chain–positive classic congenital muscular dystrophy

The group of patients with classic CMD who are laminin α2-chain–positive may well be quite heterogeneous genetically. As alluded to earlier, in the comparative studies with the laminin α2-chain–deficient patients it has become apparent that the prognosis for ambulation for the most part appears to be much better in the α2-chain–positive patients [84•,85–88]. Most achieve some form of independent ambulation. However, a recent large study from Japan examining their laminin α2-chain–positive patients without structural CNS abnormalities has shown that there might be later gradual deterioration in muscle strength when the patients reach the second and third decades of life [105•]. This would indicate that the underlying dystrophic process is progressive, albeit so slowly that initial motor development outpaces this deterioration. Because it is apparent that the laminin α2-chain–positive patients as a whole are heterogeneous, especially when ascertainment is done through analysis of the muscle biopsy [87], it is also unclear whether the disease in the Japanese patients is largely a result of the same genetic defect as in the patients in the European and American studies.

Congenital muscular dystrophies with abnormalities of brain formation and mental retardation

Fukuyama muscular dystrophy

The prototypical disease in this group is Fukuyama muscular dystrophy. In Japan, this autosomal recessive disorder is the second most prevalent muscular dystrophy after DMD. It appears to be very rare outside of Japan. In addition to neonatal hypotonia, weakness, and elevated CK levels, affected children have delayed psychomotor development, often with profound mental retardation. Seizures eventually occur in most of the cases. The muscular weakness is progressive and contractures can become prominent, so that very few patients are able to stand by the age of 4 years [106]. The mean age of death is around 10 years. CNS findings include areas of pachygyria and polymicrogyria, which are particularly well seen on three-dimensional MRI reconstructions [107]. The cerebellum is only mildly affected and hydrocephalus is rare; however, there is a relatively broad spectrum of severity [108]. Ocular involvement tends to be rare; when present, it is mild and may consist of myopia or optic atrophy. The disease gene for Fukuyama muscular dystrophy has been mapped to chromosome 9q31 by linkage analysis [109]. Although the genetic map position has recently been refined [110], the underlying genetic defect has not yet been identified. It has been shown that there is a relative deficiency of the laminin α2-chain on immunohistochemistry of diseased muscle [111–113]. This represents a secondary deficiency because the gene for the laminin α2-chain maps to chromosome 6q2.

Walker-Warburg syndrome

In Walker-Warburg syndrome, the CNS abnormalities tend to be much more severe, and there is a high degree of neonatal lethality. Type II lissencephaly is the leading cortical malformation [114•]. This is often mixed with polymicrogyria, giving the cortex a cobblestone appearance. Cerebellar hypoplasia, often associated with a Dandy-Walker anomaly, hydrocephalus, and the inconstant occurrence of encephaloceles, is also seen [115]. There are major abnormalities of the eyes, of which retinal dysplasia is the most consistent. Anterior chamber malformations, cataracts, choroidal colobomata, optic nerve hypoplasia, and microphthalmia can all occur [116]. The CNS malformation complex often dominates the clinical picture, and the constant association with a muscular dystrophy has in fact not been appreciated until recently. The histopathologic picture in muscle is indistinguishable from the other disorders in this group.

This syndrome also appears to follow autosomal recessive inheritance, but the gene has not yet been identified or localized. Toda *et al.* [117] reported a patient with an apparent Walker-Warburg phenotype occurring in a family with Fukuyama muscular dystrophy. This patient was carrying the same haplotype on chromosome 9, as did the siblings with Fukuyama muscular dystrophy.

However, this patient might also have had an unusually severe Fukuyama phenotype, as cases showing overlap to mild Walker-Warburg cases have been recognized in families with Fukuyama muscular dystrophy [108]. In contrast to Fukuyama muscular dystrophy, staining for the laminin α2-chain is normal in muscle from patients with the Walker-Warburg syndrome, suggesting that it is a distinct entity [118•]. Wewer *et al.* [119] confirmed normal α2-chain staining but also found deficient laminin β2-chain staining and abnormalities of α-sarcoglycan immunoreactivity in skeletal muscle, the significance of which is not yet clear. Some Walker-Warburg families have now been excluded genetically from the Fukuyama locus on 9q31 (W. Dobyns, Personal communication).

Muscle-eye-brain disease

Muscle-eye-brain disease is particularly prevalent in Finland, where it has occurred in a number of consanguineous pedigrees, suggesting autosomal recessive inheritance [120]. Although less severe [121], the brain abnormalities are reminiscent of those seen in the Walker-Warburg syndrome. Hydrocephalus is seen frequently. There is considerable psychomotor delay and seizures are often present. However, many patients acquire the ability to stand or walk, often followed by deterioration secondary to increasing spasticity [120]. The eye abnormalities consist of high myopia, retinal degeneration, and optic atrophy [122]. This disorder has been shown to be distinct from Fukuyama muscular dystrophy by exclusion of the 9q31 Fukuyama locus in Finnish pedigrees [123•]. However, its relationship to Walker-Warburg syndrome is not entirely clear. Haltia *et al.* [124] report weak immunostaining for the laminin α2-chain with normal laminin β2-chain staining in muscle from patients with muscle-eye-brain disease, a finding that may facilitate its distinction from Walker-Warburg syndrome.

There are other clinical phenotypes associated with CMD, sometimes restricted to a single family, that do not fit easily into the classification scheme outlined here. No completely satisfactory classification system exists at the moment, and not unlike the LGMDs, the availability of more genetic information will almost certainly be of great help to arrive at such an understanding. The genetic analysis of as many cases and families as possible, even with diverse and unusual phenotypes, will be essential to generate this knowledge.

Undoubtedly the near future will see not only the addition of even more disorders and genetic loci to the list of muscular dystrophies but also the identification of the genes that are still elusive. Along with these genetic advances should come significant insight into the pathogenesis of muscle degeneration in these muscular dystrophies—the first step to meaningful therapy for these often devastating diseases.

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Demonstration that neuronal nitric oxide synthase is associated with the complex of dystrophin-associated proteins and is reduced in DMD muscle.

Exhibit B

INVITED REVIEW

Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, is associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan which provides a linkage to the extracellular matrix component, laminin. In patients with DMD, the absence of dystrophin leads to the loss in all of the dystrophin-associated proteins, causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. This may render the sarcolemma vulnerable to physical stress. These recent developments in the research concerning the function of the dystrophin–glycoprotein complex pave a way for the better understanding of the pathogenesis of muscular dystrophies. © 1994 John Wiley & Sons, Inc.
Key words: Duchenne muscular dystrophy • dystrophin–glycoprotein complex • dystrophin-associated proteins • dystroglycan • sarcolemma
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DYSTROPHIN–GLYCOPROTEIN COMPLEX: ITS ROLE IN THE MOLECULAR PATHOGENESIS OF MUSCULAR DYSTROPHIES

KIICHIRO MATSUMURA, MD, and KEVIN P. CAMPBELL, PhD

Duchenne muscular dystrophy (DMD) is one of the most severe and common neuromuscular diseases. Although extensive research efforts have been directed toward the elucidation of the mechanism causing muscle degeneration in this devastating disease, it was not until 1986 when the causative gene was finally identified.[62] Over the last several years, biochemical investigation of dystrophin, the protein product of the DMD gene,[62] has led to the identification of a large oligomeric complex of novel sarcolemmal glycoproteins associated with dystrophin, including dystroglycan which binds the extracellular matrix component, laminin.[21,26–28,55,95] In this article, we review these recent developments in the field of skeletal muscle dystrophin research and discuss the involvement of the dystrophin-associated proteins in the molecular mechanism leading to muscle cell necrosis in DMD.

From the Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa.

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Address reprint requests to Dr. Kevin P. Campbell, Howard Hughes Medical Institute, University of Iowa College of Medicine, 400 EMRB, Iowa City, IA 52242.

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DYSTROPHIN

The entire coding sequence of dystrophin was published in 1988.[42] Dystrophin was predicted to be a rod-shaped cytoskeletal protein of 427 kd, composed of four structural domains: (1) the amino-terminal domain with high homology to actin binding regions of such actin binding proteins as α-actinin, β-spectrin, and Dictyostelium actin-binding protein 120; (2) a series of 24 repeats of 109-amino acids in the form of a triple helix; (3) a cysteine-rich domain homologous to the carboxyl-terminal domain of Dictyostelium α-actinin; and (4) the carboxyl-terminal domain with no homology to the previously described sequences at that time.[42]

Antibodies against fusion proteins or synthetic peptides were soon produced and used for the initial identification of this predicted protein.[3,17,33,80,89,95] Immunohistochemical and immunoelectron microscopic analyses localized dystrophin to the cytoplasmic face of normal skeletal and cardiac sarcolemma, and immunoblot analysis detected a protein with a molecular mass of 400 kd in normal skeletal and cardiac muscles.[3,17,33,80,89,95] Dystrophin was absent in the skeletal and cardiac muscles of DMD patients.[3,17,33,80,95]

Dystrophin was initially reported to constitute only 0.002% of the total skeletal muscle protein,[32] raising a doubt about the possibility that dystrophin could play a major structural role in skeletal

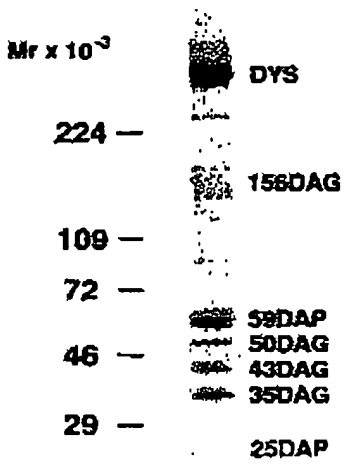

FIGURE 1A. Components of the dystrophin–glycoprotein complex separated on 3–12% SDS-PAGE. The 156DAG is not stained well with Coomassie blue due to heavy glycosylation.

muscle. However, it is now known that dystrophin constitutes 2% of total sarcolemmal protein and 5% of sarcolemmal cytoskeletal protein.[65,67] The latter figure is similar to the abundance of spectrin in brain membranes, indicating that dystrophin is a major structural component of the subsarcolemmal cytoskeleton.

Ultrastructural analysis has demonstrated that dystrophin is a rod-shaped molecule, as predicted from the primary sequence.[64,73,78] Recently, the amino-terminal domain of dystrophin was expressed as a fusion protein and shown to associate with F-actin by cosedimentation analysis.[31,90] Two putative actin-binding sites were identified by proton NMR spectroscopy of synthetic peptides corresponding to defined regions of the amino-terminal domain of dystrophin.[47] Since dystrophin is localized to the cytoplasmic face of the sarcolemma, it is presumed to interact with cytoskeletal actin such as γ-actin rather than α-actin of thin filaments in muscle cells. Morphological studies indicate that dystrophin does not distribute uniformly along the sarcolemma but is highly enriched in costameres where the Z bands are presumed to be attached to the overlying sarcolemma.[51,60,74,82]

DYSTROPHIN–GLYCOPROTEIN COMPLEX (DGC)

The mode of interaction of dystrophin with the sarcolemma was unclear until 1989 when biochemical experiments demonstrated that dystrophin is tightly associated with membrane glycoproteins.[21] Further investigation revealed that dystrophin is associated with a large oligomeric complex of novel sarcolemmal proteins comprised of a 156-kd glycoprotein (156DAG), a 59-kd protein (59DAP), a 50-kd glycoprotein which was originally called SL50 (50DAG), a 43-kd glycoprotein (43DAG), a 35-kd glycoprotein (35DAG), and a 25-kd protein (25DAP) (Fig. 1a).[26–28,36,93,94] Tight association of these proteins in the complex was demonstrated by: (1) copurification[26–28,93]; (2) cosedimentation on sucrose density gradient[26–28]; (3) coimmunoprecipitation[26–28]; (4) stoichiometric ratio[26–28,93]; (5) colocalization to the sarcolemma (Fig. 1b)[26–28]; and (6) crosslinking.[93] Dystrophin and the dystrophin-associated proteins (DAPs) also colocalize to the sarcolemma of intrafusal muscle fibers, and to the neuromuscular and myotendon junctions, two specialized regions of the sarcolemma where dense dystrophin-staining is observed (Matsumura and Campbell, unpublished results).[54,81]

Extensive biochemical analysis of the dystrophin–glycoprotein complex (DGC) indicates the following: (1) the 156DAG is an extracellular protein extractable from the membranes by pH 12 treatment; (2) the 50DAG, 43DAG, 35DAG, and 25DAP are transmembrane proteins; and (3) the 59DAP is a cytoplasmic and probably cytoskeletal protein, extractable from the membranes by pH 11

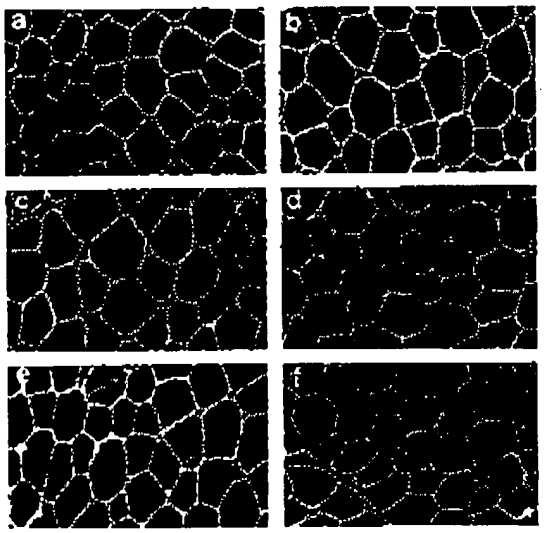

FIGURE 1B. Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in normal skeletal muscle. Immunostaining for dystrophin (a), 156DAG (b), 59DAP (c), 50DAG (d), 43DAG (e), and 35DAG (f) is shown (modified from ref. 55).

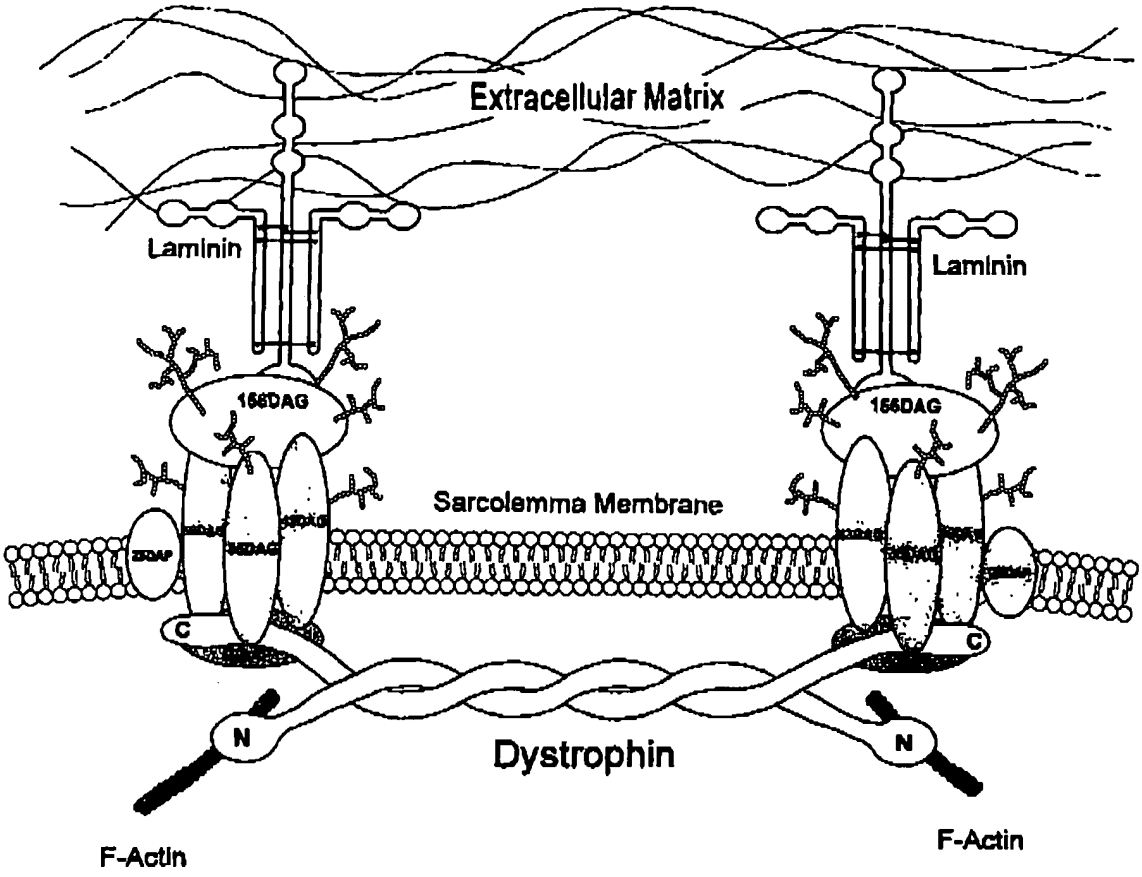

FIGURE 1C. Schematic model of the dystrophin–glycoprotein complex as a transsarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix.

treatment, like dystrophin.[28] Cosedimentation analysis demonstrates the interaction of the DGC with F-actin (Ervasti and Campbell, in press).

Which domain of dystrophin interacts with the glycoprotein complex? The C-terminal domains (cysteine-rich and carboxyl-terminal domains) were originally suggested to interact with the DAPs because of the following observations[28]: (1) the lack of significant homology between the carboxyl-terminal domain and proteins of known function except dystrophin-related protein (now called utrophin), an autosomal homologue of dystrophin[12,19,37]; (2) the conservation of the C-terminal domains of dystrophin among different species[16]; (3) the clinical observation that the phenotype of the patients with deletions in the C-terminal domains is severe[9,42,49]; and (4) the results of immunogold labeling studies.[23,24] More recently, the results of limited calpain digestion of the DGC demonstrated that the DAPs-binding site was confined to the cysteine-rich and the first half of the carboxyl-terminal domains.[83] On the other hand, dystrophin lacking the C-terminal domains was reported to be localized properly to the sarcolemmal region in unique patients with DMD.[14,30,34,76] This led to a speculation that the C-terminal domains are not essential for the interaction of dystrophin with the sarcolemma.[14,30,34,76] However, the possibility that truncated dystrophin with an intact amino-terminal domain may properly localize to the sarcolemmal region by associating with other subsarcolemmal cytoskeletal components such as γ-actin, even when it is not associated with the DAPs, was not addressed. This question will be discussed further in the section "DMD Patients Lacking the C-Terminal Domains of Dystrophin."

Recently, dystrophin was reported to be associated with a postsynaptic protein with molecular

mass of 58 kd in Torpedo electric tissue, which is derived embryologically from immature striated muscle and retains many similarities to mammalian skeletal muscle.[19] Another postsynaptic protein with molecular mass of 87 kd, which shares homology with the C-terminal domains of dystrophin, was also shown to be associated with this 58-kd protein.[19,88] Immunohistochemical analysis indicates the presence of a mammalian skeletal muscle protein which shares immunological homology with the 58-kd Torpedo protein.[19]

PRIMARY STRUCTURE OF DYSTROGLYCAN (43DAG/156DAG)

In order to understand the function of the DAPs, the primary structure of each component had to be clarified. A single cDNA encoding two of the DAPs, the 43DAG and 156DAG, was isolated and characterized.[35] Posttranslational processing of a 97-kd precursor protein translated from a 5.8-kb mRNA results in these two proteins.[35] Consistent with the aforementioned biochemical data, the carboxyl-terminal portion of the precursor protein processed into the 43DAG has three potential *N*-glycosylation sites, a single potential transmembrane domain and a 120-amino-acid–long cytoplasmic tail.[35] The amino-terminal portion of the precursor protein corresponding to the 56-kd core protein of the 156DAG has no transmembrane domain but one potential *N*-glycosylation site and many potential *O*-glycosylation sites.[35] Carbohydrate moieties constitute almost two-thirds of the molecular mass, suggesting that the 156DAG may be a proteoglycan.[35] Heavy glycosylation is presumed to explain the high resistance of the 156DAG to proteolysis.[28] Based on the glycosylated nature and the association with dystrophin, the 43DAG/156DAG was named dystroglycan.[35]

LAMININ-BINDING PROPERTIES OF DYSTROGLYCAN: DGC IS A TRANSSARCOLEMMAL LINKER BETWEEN THE SUBSARCOLEMMAL CYTOSKELETON AND THE EXTRACELLULAR MATRIX

The 156-kd dystroglycan has been shown to bind the extracellular matrix component, laminin.[35] This binding is inhibited by high salt, divalent-cation chelating agent (EDTA) or heparin (Ervasti and Campbell, in press). The 156-kd dystroglycan is a highly specific laminin receptor: it does not bind other well-characterized extracellular matrix components such as fibronectin, collagen I, collagen IV, entactin, or heparan sulphate proteoglycan (Ervasti and Campbell, in press). Dystrophin, all of the DAPs, and laminin colocalize to the sarcolemma in skeletal muscle, and to the sarcolemma and transverse tubules in cardiac muscle.[41]

These findings indicate that the DGC is a transsarcolemmal linker between the subsarcolemmal cytoskeleton and the extracellular matrix (Fig. 1c). The DGC is expected to provide a structural support to the sarcolemma and, indeed, could be a unique plasma membrane-supporting mechanism which has developed in striated muscle, a tissue which undergoes both extreme contraction and stretch.[91] In addition to this structural role, the DGC may have far more diverse biological functions such as signal transduction and regulation of the intracellular calcium concentration.

Does the DGC or a homologous complex exist in nonmuscle tissues and, if so, what is its function? Northern blot analysis has demonstrated that dystroglycan mRNA is expressed not only in skeletal, cardiac, and smooth muscles but also in nonmuscle tissues such as brain, lung, liver, and kidney which do not express dystrophin to any significant extent.[35] A cell surface laminin-binding protein with molecular mass of 120 kd was purified from brain and shown to have the primary sequence identical with the 156-kd dystroglycan.[48] The difference in size of the 156-kd dystroglycan from skeletal muscle and brain suggests a different level of glycosylation of the protein between these two tissues, which could reflect different functions of the same gene product in different tissues. Difference in size of the 156-kd dystroglycan is also found among skeletal muscle, diaphragm, cardiac muscle, smooth muscle, lung, and kidney (Ibraghimov-Beskrovnaya and Campbell, personal communication).

At present, the cellular distribution of dystroglycan in nonmuscle tissues is not known. It is also unclear if dystroglycan is associated with dystrophin or dystrophin isoforms/homologues in these tissues. Recently, a novel DMD gene product with molecular mass of 71 kd was identified in nonmuscle tissues, including brain, lung, liver, and kidney.[8,15,44] Another DMD gene product with molecular mass of 116 kd was identified in the peripheral nerve.[20] Since these two proteins share homologous C-terminal domains with full-size dystrophin, they could associate with the DAPs in nonmuscle tissues.

The primary structure, function, and tissue distribution of the DAPs other than dystroglycan are unknown. However, partial amino acid sequence analysis indicates that each of the DAPs is a novel protein. Characterization of all of the DAPs at both molecular biological and biochemical levels is es-

sential for the better understanding of not only the structural organization and function of the DGC in skeletal muscle but also the identification of the homologous complexes in nonmuscle tissues.

THE ROLE OF THE DGC IN THE MOLECULAR PATHOGENESIS OF THE mdx MOUSE

The elucidation of the precise mechanism by which the absence of dystrophin leads to muscle cell necrosis is a prerequisite for the development of effective therapies for DMD, the ultimate goal of DMD research. In this respect, the dystrophin-deficient mdx mouse is a good animal model for biochemical investigations.

Immunohistochemistry revealed that all of the DAPs were drastically reduced in the sarcolemma of mdx mice.[69] Immunoblot analysis showed approximately 80–90% reduction in all of the DAPs in mdx skeletal muscle membranes compared with normal membranes.[69] These results were independent of the age of the animals and the severity of degeneration of individual muscle fibers, indicating that the loss of the DAPs is a direct consequence of the absence of dystrophin and not due to the nonspecific secondary effects of muscle fiber degeneration.[69] This hypothesis is also supported by the finding that all of the DAPs are well preserved in *dy/dy* mice which have normal expression of dystrophin but have severe dystrophy.[69]

Is the synthesis of the DAPs reduced or is the degradation increased in mdx mice? Northern blot analysis revealed the normal production of the dystroglycan mRNA in mdx skeletal muscle.[35] This suggests that the DAPs are synthesized but may not be properly assembled and/or integrated into the sarcolemma or may be degraded in the absence of dystrophin.[35]

What is the status of the residual 10–20% of the DAPs in mdx skeletal muscle? The results of sucrose density gradient centrifugation and immunoprecipitation experiments suggest the presence of four subfractions of the DAPs in mdx skeletal muscle: (1) a complex of the 156DAG and 43DAG; (2) a complex of the 50DAG and 35DAG; (3) unassociated 59DAP; and (4) the DAPs associated with utrophin (discussed later.)[54] Since the DAPs associated with utrophin constitute less than 20–30% of the residual DAPs, most of the residual DAPs do not serve a function in the linkage of the subsarcolemmal cytoskeleton to the extracellular matrix.[54] This suggests that the actual disruption of this linkage in mdx skeletal muscle is far more severe than what one expects from the level of the DAPs in the sarcolemma as revealed by immunohistochemistry.

MOLECULAR PATHOGENESIS OF DMD AND RELATED DISEASES

DMD. The structural organization of the DGC (Fig. 1c) suggested that the absence of dystrophin may disrupt the linkage of the DAPs to the subsarcolemmal actin–cytoskeleton in DMD skeletal muscle. This could lead to the dysfunction of the DGC and/or the loss of the DAPs in the sarcolemma, in analogy to other diseases involving cytoskeletal proteins, such as hereditary elliptocytosis,[2] in which the deficiency in one component of the membrane cytoskeleton leads to the loss of the other components.

Immunohistochemical analysis revealed a drastic reduction in all of the DAPs in DMD patients of various ages (Fig. 2).[70] The loss of the DAPs is considered a direct consequence of the absence of dystrophin and not due to the nonspecific secondary effects of muscle degeneration, based on the following observations: (1) all of the DAPs are preserved in a variety of other neuromuscular diseases where muscle fiber necrosis and degeneration occur; (2) the loss of the DAPs is common in all DMD patients, irrespective of age; (3) the loss of the DAPs is found in all muscle fibers, independent of the severity of degeneration; (4) the abundance of many other glycoproteins is not affected in DMD muscle; and (5) other proteins including the membrane cytoskeletal protein spectrin remain well preserved in DMD.[70]

Based on these results and the structural organization of the DGC, we proposed that the disruption of the DGC could play a key role in the cascade of events leading to muscle cell necrosis in DMD.[70] The absence of dystrophin causes the disruption of the linkage of the DAPs to the subsarcolemmal actin–cytoskeleton, which leads to a drastic reduction in all of the DAPs. The resulting disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix may lead to sarcolemmal instability and eventually to muscle cell necrosis.[70] This may be the case, especially during muscle contraction which may cause physical breaks or tears of the sarcolemma. This hypothesis is quite consistent with the reported morphological abnormalities in the sarcolemma of DMD patients.[16,22,77,79]

DMD Patients Lacking the C-Terminal Domains of Dystrophin. Dystrophin lacking the C-terminal domains was reported to be localized properly to the sarcolemma in unique patients afflicted with DMD.[14,30,34,76] Despite the proper intracellular localization of truncated dystrophin, the phenotype of these patients was quite severe. This indicated

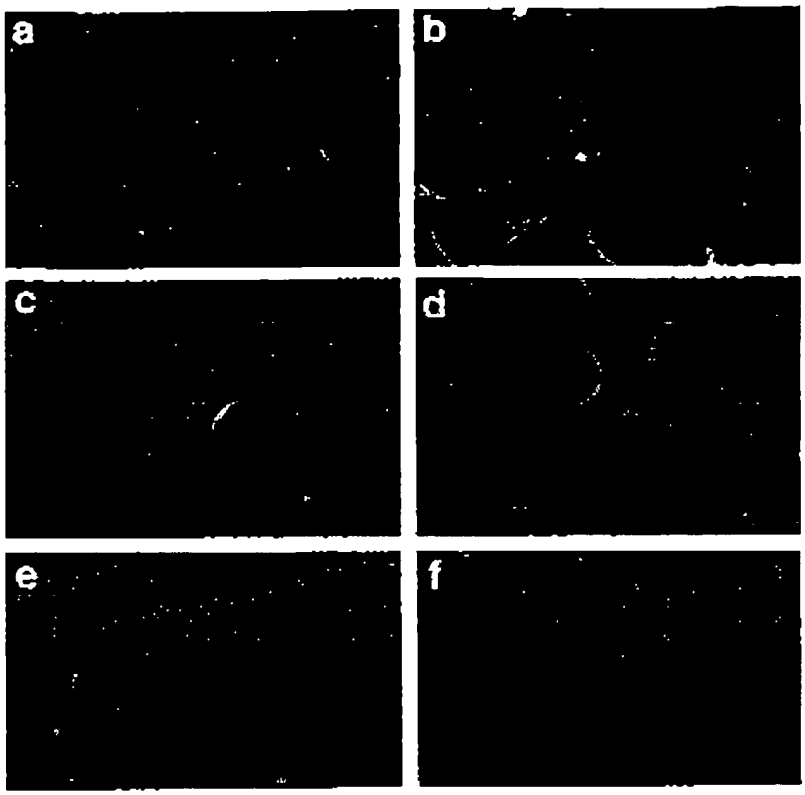

FIGURE 2. Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in skeletal muscle from a DMD patient. Immunostaining for dystrophin (a), 156DAG (b), 59DAP (c), 50DAG (d), 43DAG (e), and 35DAG (f) is shown (modified from ref. 55). Dystrophin is absent and all of the dystrophin-associated proteins are greatly reduced in the sarcolemma.

that the C-terminal domains were likely to be very important in the function of dystrophin.[14,30,34,76]

Recently, the status of the DAPs was studied in similar patients. Immunohistochemistry revealed that all of the DAPs were drastically reduced in the sarcolemma even though dystrophin lacking the C-terminal domains was properly localized to the sarcolemmal region.[58] The results suggest that the DAPs-binding site is missing in these patients, and thus, are consistent with the observation that the C-terminal domains are essential for interaction with the DAPs.[83] The loss of the DAPs in the sarcolemma causing the disruption of the linkage between the subsarcolemmal cytoskeleton and extracellular matrix is presumed to be the cause of the severe phenotype of these patients.[58]

Symptomatic DMD Carriers. Dystrophin deficiency is found in some muscle fibers and is speculated to cause muscle fiber degeneration in symptomatic DMD carriers.[4,59] To test this hypothesis, it was important to know the status of the DAPs in these individuals. Immunohistochemistry showed that all of the DAPs were lost in the sarcolemma of dystrophin-deficient muscle fibers, while they were well preserved in dystrophin-positive fibers, in symptomatic DMD carriers (Fig. 3) (Sewry et al., manuscript in preparation).[57] This indicates that the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix is disrupted in dystrophin-deficient fibers.[57] Thus, the same sarcolemmal instability as in the case of DMD may be responsible for the muscle fiber degeneration in symptomatic DMD carriers.

Becker Muscular Dystrophy (BMD). Immunohistochemistry has shown reduced and/or patchy dystrophin staining along the sarcolemma, and immunoblot analysis has detected dystrophin of abnormal size and/or reduced quantity.[5,6,55] How-

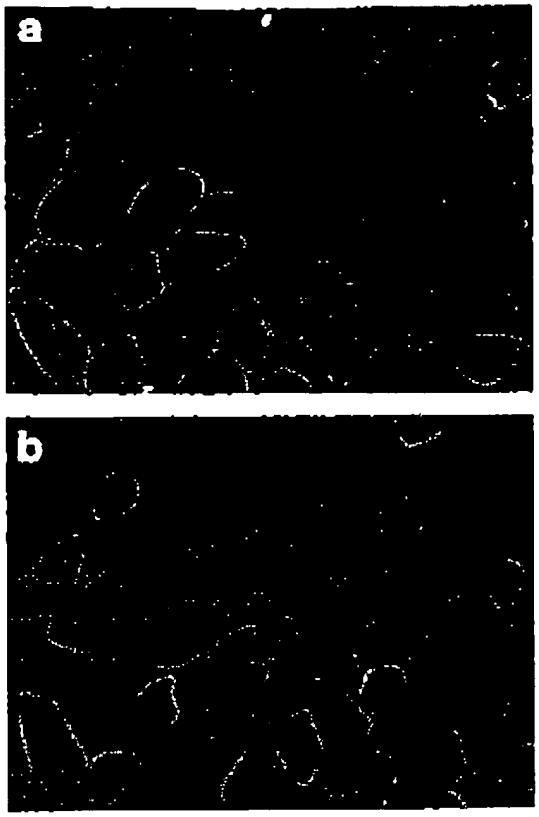

FIGURE 3. Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in skeletal muscle from a symptomatic DMD carrier. Immunostaining for dystrophin (a) and 50DAG (b) is shown. Dystrophin-associated proteins (exemplified by 50DAG) are greatly reduced in the dystrophin-deficient muscle fibers, while they are well preserved in the dystrophin-positive fibers.

ever, the mechanism by which these reported abnormalities of dystrophin lead to muscle fiber degeneration of BMD is unclear. Analysis of the status of the DAPs in BMD patients having various mutations in the dystrophin gene would be important to answer these questions. This study could give us information about the domains of dystrophin essential for the interaction with the DAPs, and would also have significant implications on the design of dystrophin minigenes[75] in the potential gene therapies for DMD.

Immunohistochemistry showed a correlation between the reduction in dystrophin and DAPs staining in BMD patients having in-frame deletions in the rod domain of dystrophin (Matsumura et al., in press). The reduction in the DAPs was milder than in typical DMD patients or the DMD patients lacking the C-terminal domains of dystrophin, indicating that the rod domain is not crucial for the interaction with the DAPs. This suggests that in-frame mutations of the dystrophin gene having no effects on the interaction with the DAPs will not result in a significant loss of the DAPs and/or the disruption of the linkage to the extracellular matrix. However, dystrophin with defects in the rod domain may not have a normal function or may be unstable, and this may lead to a mild reduction in the density of the DGC. This could explain the mild phenotype of these BMD patients. In patients with mutations in the amino-terminal domain of dystrophin, on the other hand, the anchorage of the DGC to the subsarcolemmal actin–cytoskeleton may be disrupted due to the loss or defects of the actin-binding activity of dystrophin. Analysis of the DAPs in these patients would be interesting.

Autosomal Muscular Dystrophies with DMD-Like Phenotype. Recent discoveries about the structural organization of the DGC raised a possibility that a primary defect of a DAP could be the cause of autosomal muscular dystrophy. So far, two autosomal diseases have been reported to show abnormalities of the DAPs.

Severe Childhood Autosomal-Recessive Muscular Dystrophy (SCARMD). Specific deficiency of the 50DAG was demonstrated in the patients afflicted with a severe childhood autosomal recessive form of muscular dystrophy which is prevalent in North Africa (Fig. 4).[53] Patients with SCARMD present with DMD-like symptoms despite the normal expression of dystrophin.[11,12,53] Since the 50DAG deficiency is common to both DMD and SCARMD, it is presumed to be playing an important role in the molecular pathogenesis leading to muscle cell necrosis in these two diseases.[53] In contrast to DMD, where the absence of dystrophin causes a secondary reduction in all of the DAPs and the disruption of the DGC, the deficiency of the 50DAG may cause a dysfunction of the DGC in SCARMD.[53] Although the deficiency of the 50DAG characterizes the early stages of this disease, other components of the DGC could also be affected in the advanced stages.[53]

The initial identification of the 50DAG deficiency was made in 1 Lebanese and 3 Algerian patients.[53] Why is SCARMD prevalent in North Africa? Although the high rate of consanguinity in this region could be the cause, this disease may be specific to Arab populations. Recently, two studies

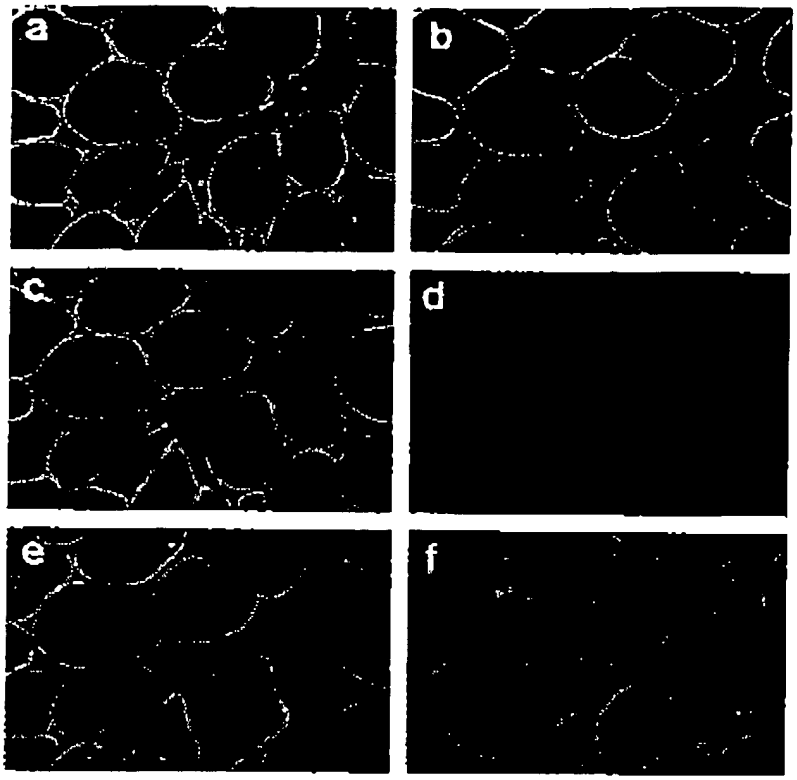

FIGURE 4. Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in skeletal muscle from a SCARMD patient. Immunostaining for dystrophin (a), 156DAG (b), 59DAP (c), 50DAG (d), 43DAG (e), and 35DAG (f) is shown. Although dystrophin, 156DAG, 59DAP, and 43DAG are well preserved, 50DAG is drastically reduced and 35DAG is slightly reduced in the sarcolemma.

were carried out to know if this disease exists in the non-Arab populations. In a study of European patients afflicted with severe childhood muscular dystrophy, 1 Italian, 1 Greek, and 3 French patients were found to be deficient in the 50DAG despite the near-normal presence of dystrophin and the other DAPs (Fardeau et al., submitted). In a Brazilian study, 4 negroid patients with the 50DAG deficiency were identified (Zatz et al., submitted). Thus, SCARMD exists in various populations. All of these non-Arab patients had been diagnosed as DMD/BMD on clinical grounds until the immunochemical test revealed the deficiency of the 50DAG instead of dystrophin. Interestingly, consanguinity was negative in all of the European patients. This suggests that the high rate of consanguinity may be the cause of high prevalence of SCARMD in North Africa, even though this disease may not be specific to Arab populations. Whether the 50DAG deficiency exists in the North American or Asian populations remains to be investigated.

With increasing numbers of patients identified, a clearer picture is emerging for the phenotype of 50DAG deficiency. It resembles the phenotype of severe BMD or so-called outliers in many respects. It can be summarized as the following: (1) both sexes are affected equally; (2) weakness of the lower extremities begins between 5 and 10 years of age; (3) calf hypertrophy is common in the early stages; (4) some patients become wheelchair bound as early as 10 years of age; (5) the serum CK value is elevated to 50 times the normal upper limit in the early stages; (6) electromyography and muscle histology reveal myopathic changes which resemble but are milder than those of DMD; (7) early death due to cardiomyopathy can occur; (8) severity of the symptoms vary greatly among both unrelated and related patients; and (9) mental retardation is absent.

The primary defect causing the deficiency of the 50DAG in SCARMD is unknown. It could be due to a primary defect in the structure or expression of the gene for this protein or a secondary effect of an unknown primary defect. Molecular biological and linkage analysis will be needed for the elucidation of the primary cause of SCARMD. Recently, the defective gene responsible for Tunisian autosomal recessive Duchenne-like muscular dystrophy (DLMD) was mapped to the pericentromeric region of chromosome 13q by linkage analysis.[13] It is crucial to clarify the relationship between the DLMD gene and the 50DAG.

Fukuyama-Type Congenital Muscular Dystrophy (FCMD). FCMD is a severe autosomal recessive muscular dystrophy prevalent in Japan.[29,65] The phenotype of FCMD consists of muscular dystrophy and brain anomaly.[29,65] In most cases, dystrophin is expressed at near-normal level in this disease.[5,7,52] Recently, the abnormal expression of the DAPs was reported in FCMD skeletal muscle.[55] The DAPs staining was reduced in the sarcolemma in a number of muscle fibers despite the near-normal expression of dystrophin (Fig. 5).[55] Muscle fibers with abnormally intense staining of the sarcolemma or with diffuse cytoplasmic staining were also observed.[55]

Genetic observations have suggested a possible interaction between dystrophin and the putative FCMD gene product.[10] Based on the phenotype, the FCMD gene product is expected to be expressed in both muscle and brain. Interestingly, the abnormality of the expression of the 43-kd dystroglycan was prominent in FCMD muscle (Fig. 5).[55] Since dystroglycan is expressed in both muscle and brain,[35] these findings suggest the dystroglycan gene as a candidate gene for FCMD mutations.

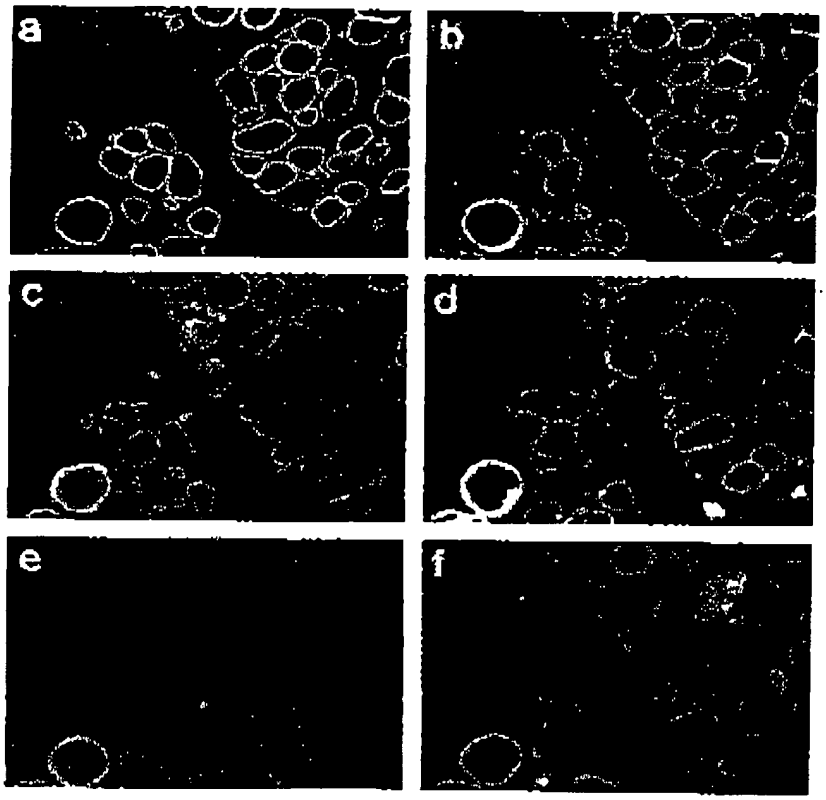

FIGURE 5. Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in skeletal muscle from a FCMD patient. Immunostaining for dystrophin (a), 156DAG (b), 50DAP (c), 50DAG (d), 43DAG (e), and 35DAG (f) is shown (modified from ref. 55). Although dystrophin is well preserved, the dystrophin-associated proteins are generally reduced in the sarcolemma. Muscle fibers with abnormally intense staining of the sarcolemma or diffuse cytoplasmic staining for the dystrophin-associated proteins are also observed.

DIAGNOSIS OF MUSCULAR DYSTROPHIES

The findings described above indicate that the status of the DAPs in the sarcolemma shows a good correlation with the severity of the clinical symptoms in certain muscular dystrophies, including DMD, BMD, DMD carriers, SCARMD, and possibly FCMD (Table 1). Thus, the immunochemical analysis of the DAPs, in addition to dystrophin, may be effective for the accurate diagnoses of these diseases. This is especially the case for the diagnosis of male sporadic patients who are afflicted with severe muscular dystrophy and have reduced amount of normal-sized dystrophin in the immunoblot analysis. According to the standard diagnostic criteria, they would be diagnosed as severe BMD/outlier.[39] However, a fraction of these patients could be afflicted with SCARMD instead, since the phenotype of SCARMD is very close to that of severe BMD/outlier and dystrophin could be reduced in the advanced stages of SCARMD.[53] This indicates that the immunochemical analysis of the 50DAG is necessary for the differential diagnosis of these patients.

So far immunohistochemical abnormalities of the DAPs have not been found in the following diseases: limb-girdle muscular dystrophy, myotonic dystrophy, facioscapulohumeral muscular dystrophy, oculopharyngeal muscular dystrophy, non–Fukuyama-type congenital muscular dystrophy, and spinal muscular atrophy (Matsumura et al., submitted). This does not necessarily exclude a possibility of dysfunction(s) in the components of the DAPs undetected by the current immunohistochemical methods.

Table 1. Correlation between the phenotype and the status of the expression of dystrophin and the dystrophin-associated proteins (DAPs).

Phenotype	Dystrophin	DAPs
Normal	+	+
DMD	–	Severe reduction
DMD	+ (Lacking C-terminal domains)	Severe reduction
Symptomatic DMD carrier	Mosaic	Mosaic
BMD	Reduced and/or patchy (intact C-terminal domains)	Reduced and/or patchy
SCARMD	+	50DAG deficiency
FCMD	+	Abnormal expression

UTROPHIN–GLYCOPROTEIN COMPLEX

Utrophin is an autosomal homologue of dystrophin.[49,87] While utrophin is ubiquitously expressed, it is localized exclusively to the neuromuscular junction in adult skeletal muscle.[38–40,50,54,68,84] Utrophin is associated with the DAPs or their homologues in skeletal muscle,[54] suggesting that the utrophin–glycoprotein complex could be playing an important role in the formation and maintenance of the neuromuscular junction.

In contrast to normal muscle, utrophin appears to spread out of the neuromuscular junction and be expressed throughout the sarcolemma in muscle from DMD patients and mdx mice.[39,54,84,85] In mdx mice, this phenomenon seems most prominent in the small-caliber skeletal and cardiac muscles which are relatively free from degeneration.[54] Furthermore, in these muscles of mdx mice, the dystrophin/utrophin-associated proteins are well preserved in the sarcolemma compared to the large skeletal muscles such as quadriceps muscle.[54] In the large skeletal muscles of mdx mice, less than 20–30% of the residual DAPs are associated with utrophin.[54] Since the residual DAPs in these muscles of mdx mice are equivalent to only 10–20% of the normal level,[60] the DAPs potentially serving as a link between the subsarcolemmal cytoskeleton and extracellular matrix is presumed to be as low as 2–6% of the normal level in these muscles of mdx mice. Thus, the upregulation of utrophin could have compensatory effects for dystrophin deficiency, but the level of upregulation in the large skeletal muscles of mdx mice might not be high enough to fully compensate for the absence of dystrophin.

THERAPEUTIC IMPLICATIONS OF THE DAPs FOR DMD

The finding that all of the DAPs, including the laminin-binding dystroglycan and the 50DAG, whose deficiency alone causes severe muscular dystrophy, are lost in the DMD sarcolemma raised a serious question concerning the efficacy of the potential therapies for DMD, such as myoblast transfer therapy[37,63,71] or dystrophin gene therapy.[1,25,45,75,92] The success of such therapies will depend not only on the replacement of dystrophin but also on the restoration and stabilization of the DAPs in the sarcolemma. Since dystrophin can properly localize to the sarcolemmal region without interaction with the DAPs as described above, localization of dystrophin to the sarcolemmal region after these therapies does not necessarily mean that all of the DAPs are restored in the sarcolemma.

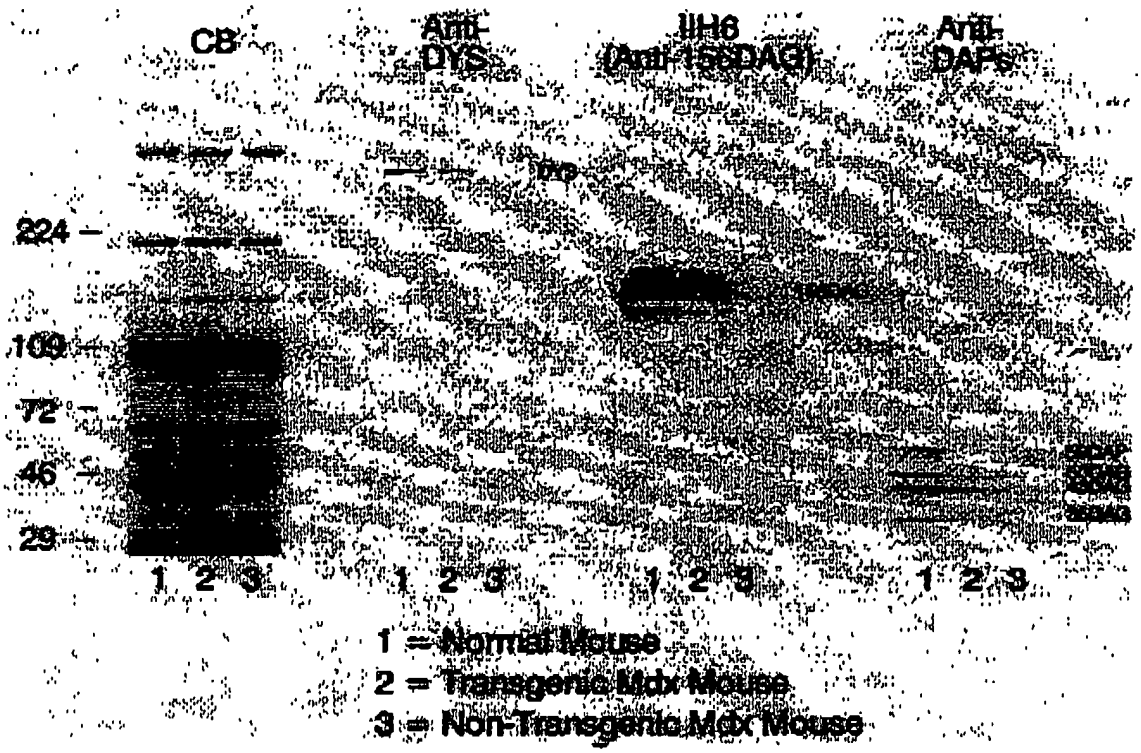

FIGURE 6. Immunoblot analysis of dystrophin and the dystrophin-associated proteins in skeletal muscle membranes from normal mouse (lane 1), mdx mouse transgenic for the full-size dystrophin gene (lane 2), and nontransgenic mdx sibling (lane 3).[56] Dystrophin and the dystrophin-associated proteins are restored to approximately 40–50% of the normal level in this transgenic mdx mouse.

Recently the status of the DAPs was analyzed in mdx mice transgenic for the full-size dystrophin gene.[56] All of the DAPs were restored in the sarcolemma of dystrophin-positive muscle fibers, while the DAPs remained reduced in dystrophin-negative fibers, in transgenic mdx mice.[56] Both immunohistochemical and immunoblot analyses demonstrated a good correlation between the level of restoration of the DAPs and the expression of dystrophin (Fig. 6).[56] The results suggest that the gene transfer therapy of dystrophin could be effective in restoring all components of the DGC and, presumably, in correcting the molecular defects. Immunochemical analysis of the status of the DAPs in the sarcolemma will be useful for the evaluation of these potential therapies.

Another intriguing therapeutic approach for DMD is the utilization of a protein which could substitute for dystrophin in skeletal muscle. One such candidate is utrophin as described above. If the expression of utrophin could be upregulated by a genetic or pharmacological manipulation in DMD muscle, it could be beneficial for the prevention of muscle degeneration. A similar therapeutic approach was reported for the β-globin diseases.[72] Butyrate, a natural fatty acid which is known to stimulate the synthesis of the fetal isoform of globin (γ-globin), was intravenously administered to patients with β-globinopathies.[72] Both the proportion of reticulocytes producing hemoglobin F and the level of γ-globin mRNA increased in response.[72] These findings suggest that pharmacological agents which stimulate the expression of utrophin could have potential therapeutic value for DMD.

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Dystrophin-associated proteins in muscular dystrophy

Eijiro Ozawa*, Mikiharu Yoshida, Atsushi Suzuki, Yuji Mizuno, Yasuko Hagiwara and Satoru Noguchi

National Institute of Neuroscience, NCNP, 4-1-1, Ogawahigashi-cho, Kodaira, Tokyo 187, Japan

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Dystrophin-associated proteins (DAPs) are classified into a few groups, namely, those comprising of dystroglycan complex, sarcoglycan complex, syntrophin complex and others. Subsarcolemmal actin filaments are connected to laminin in the basement membrane through dystrophin and the dystroglycan complex. This system may function to protect muscle fibers from mechanical damage. Furthermore, the sarcoglycan complex is associated with the system. Defects in the components of the protection system or the sarcoglycan complex or both are characteristically found in various muscular dystrophies. The roles of the syntrophin complex are meagerly understood. In this review, the possible roles of laminin, DAPs and dystrophin in each dystrophy are explained.

INTRODUCTION

In addition to interest in dystrophin, defects of which give rise to Duchenne and Becker muscular dystrophy (DMD and BMD, respectively), much interest of researchers into muscular dystrophy has recently focused on dystrophin-associated proteins (DAPs) (1), defects of which result in various muscle dystrophies. The purpose of the present review is to understand the dystrophic changes in muscles in relation to DAPs.

MOLECULAR ARCHITECTURE OF DYSTROPHIN AND DAPS

Dystrophin-associated proteins

When dystrophin, a long, slender protein present in the subsarcolemmal cytoskeletal network, is solubilized from sarcolemmal fraction with a detergent, digitonin, it has been found to be associated with several proteins. At first, these DAPs were considered to be a single unit forming an oligomeric complex. However, they were revealed to be comprised of three complexes and classified as follows both biochemically and pathologically (2; for other references see below; see also Fig. 1).

I. Glycoprotein complex
A. Dystroglycan complex
α-dystroglycan (156DAG)
β-dystroglycan (43DAG)

These are translated from a single mRNA (human gene locus: 3p21) as a 97 kDa protein which is then processed into two peptides (3) which are closely associated (2). They are ubiquitously expressed in various tissues.

B. Sarcoglycan complex
adhalin (50DAG: 17q21) (4–6)
A3b (2)
35DAG

The sarcoglycan complex is expressed specifically in skeletal and cardiac muscles (7,8).

II. Syntrophin complex
α-syntrophin (59DAP)
Fairly muscle-specific (9,10).
β1-syntrophin (59DAP: 8q23–24) (11)
Rather ubiquitously expressed (11).
β2-syntrophin (59DAP: 16q23)
Localized at the neuromuscular junction (9,12).
A0

III. Unclassified
A5 or 25DAP and others

A3b (43 kDa protein) and 43DAG (β-dystroglycan) have similar molecular masses but different isoelectric points, peptide maps, immunoreactivities, complex formation patterns, and tissue distribution patterns (2). Despite these differences, they are sometimes mixed up (13).

Basal lamina

The basal lamina is composed of a network including collagen fibers, laminin, entactin/nidogen, and heparan sulfate proteoglycans. Forming a thick sheet, it covers each muscle fiber in close contact with sarcolemma and serves to protect the fibers from mechanical damage. In many loci corresponding to

*To whom correspondence should be addressed

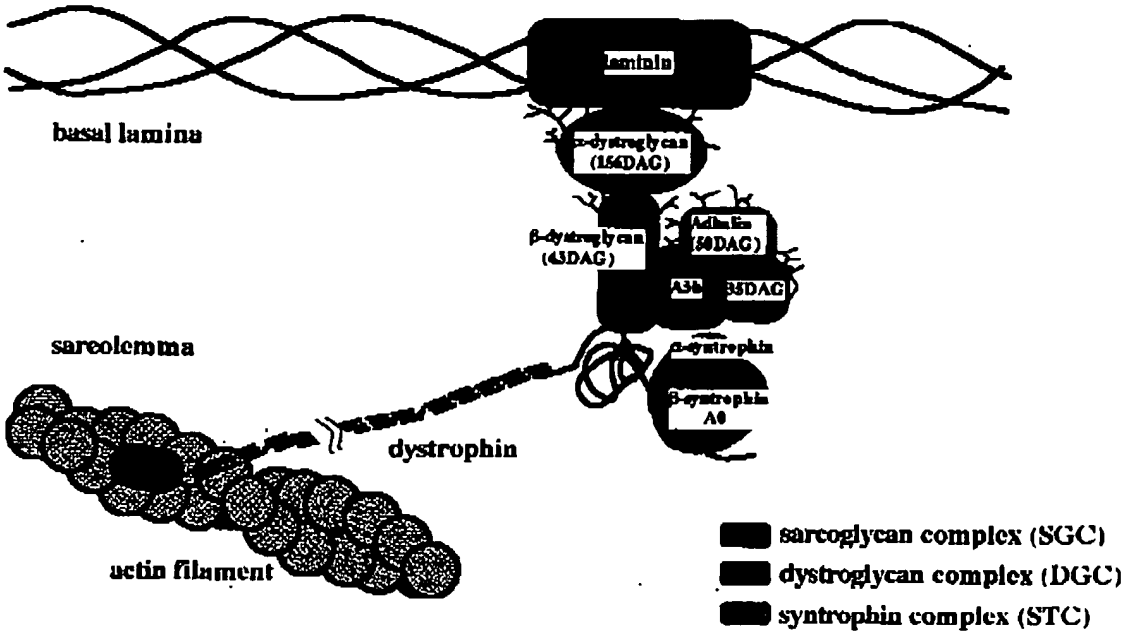

Figure 1. Molecular architecture of dystrophin and other molecules (revised from refs 16 and 53).

costameres, the basal lamina is fixed with sarcolemma and the two do not slide against each other.

Dystrophin-axis

α-dystroglycan, an extracellular protein, binds to laminin probably via its sugar chains (14) (Fig. 1). Laminin is a component of the basal lamina comprised of three subunits α, β and γ. The α subunit binds to α-dystroglycan. In most regions of the basal lamina of muscle fibers, laminin 2 (α2β1γ1) is present.

α-dystroglycan also binds to β-dystroglycan (2), a transmembranous protein (4). Inside the cell, β-dystroglycan, in turn, binds to the cysteine-rich domain and the first half of the C-terminal domain of dystrophin which are collectively called the dystroglycan-binding domain (D-domain) (Fig. 2) (15,16).

At their N-terminal regions, dystrophin molecules bind to actin-filaments which are abundantly present as components of the cytoskeleton which forms the subsarcolemmal undercoat.

In short, there is a connecting axis anchored by laminin between the subsarcolemmal cytoskeleton and extracellular matrix, which is tentatively called the 'dystrophin-axis' (actin–dystrophin–dystroglycan complex; Fig. 3; normal). The axis present at costameres on the sarcolemma may, therefore, serve to construct a cell-matrix adherens junction to fix the sarcolemma to the mechanically tough basal lamina forming a protection system for the sarcolemma. There is no evidence which suggests that the sarcoglycan complex and syntrophin complex directly contribute structurally to the axis (16).

Utrophin, a dystrophin homologue, has actin-binding (A) and D domains (Fig. 2) highly homologous (80% at the amino acid level) to those of dystrophin (17), and also binds to actin (18) and probably to the dystroglycan complex (19) at A and

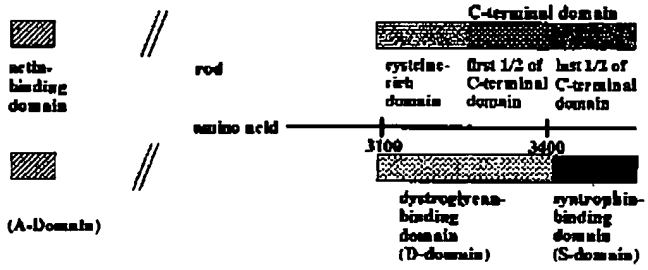

Figure 2. A new model of the domain organization of dystrophin. The top bar denotes dystrophin with the original nomenclature of the domains. The bottom bar denotes dystrophin with the revised domain border and domain names (revised from ref. 21).

D domains, respectively. Therefore, it is likely that utrophin and these proteins form a 'utrophin-axis' in various cells (actin–utrophin–dystroglycan complex), as utrophin and the dystroglycan complex are expressed in various cells as well as actin and laminin 1 (α1β1γ1). In addition, the utrophin-axis may be formed in DMD muscle.

Sarcoglycan complex

The sarcoglycan complex, which is a complex of membrane-integrated proteins (2,4,6,20), is considered to be fixed to the dystrophin-axis by lateral association with the dystroglycan complex (15,16) (Fig. 1). The exact binding site of the sarcoglycan complex to dystroglycan complex, however, has not been identified. The cDNA of adhalin was cloned and shown to include a region coding for a transmembrane domain (4,6). The cDNAs of the other two proteins have not been cloned.

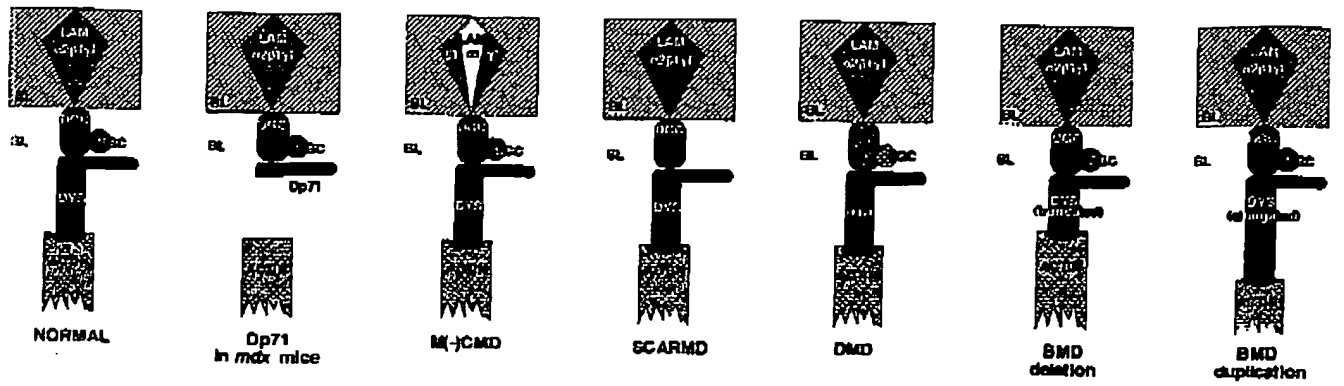

Figure 3. Simplified models of the molecular architecture of dystrophin and utrophin axes in normal and dystrophic muscle (69). LAM: Laminin, DGC: dystroglycan complex, SL: sarcolemma, SGC: sarcoglycan complex, DYS: dystrophin, ACTIN: actin filament, UTR: utrophin. The syntrophin complex is omitted from these models, because its significance in dystrophic pathogenesis is not well understood. M(–) CMD: merosin-negative CMD. Note that the dystroglycan complex is fairly well preserved and the sarcoglycan complex is drastically reduced in DMD and that dystrophin is replaced by utrophin to some extent.

The functions of the sarcoglycan complex are not known, although crucial roles in the pathogenesis of muscular dystrophy have been suggested.

Syntrophin complex binding to dystrophin

The syntrophin complex (13) binds to the second half of the C-terminal domain of dystrophin (S-domain, Fig. 2). More precisely, α-syntrophin binds to amino acid positions from 3444 to 3494, a region corresponding to the 3′-half of exon 73 and 5′-half of exon 74 (21,13), and A0 and/or β1-syntrophins bind to the 3′-half of exon 74 and 5′-half of exon 75 (21). The binding site of β1-syntrophin more exactly may be restricted to exon 74 (22). These loci correspond to the splice-prone region of dystrophin.

No study on the interaction of syntrophin complex with other DAPs has been published.

VARIOUS MUSCULAR DYSTROPHIES WHICH AFFECT THE BASAL LAMINA, DYSTROPHIN-AXIS AND SARCOGLYCAN COMPLEX

Recent studies have shown that defects in components of the basal lamina, dystrophin-axis and/or sarcoglycan complex cause the dystrophic changes in muscle. Each defect gives rise to a somewhat different phenotype.

Lesions of protection system

Dystrophin-axis lesions. Very recently, it was reported that Dp71 expression in muscles of transgenic *mdx* mice did not result in alleviation of the dystrophic phenotype (23,24). These mice have a nonsense point mutation in the rod domain of the DMD gene and lack dystrophin. Thus, *mdx* muscles undergo dystrophic changes. Dp71 molecules are a product of the DMD gene but are composed almost exclusively of D and S domains (25; Fig. 2). Although Dp71 molecules, when overexpressed in *mdx* mice, are localized at the sarcolemma, and dystroglycan complex and sarcoglycan complex are restored, pathological findings of muscle and creatine kinase activity in serum remained unchanged. As Dp71 molecules do not have A domain and do not bind to the actin filaments, the dystrophin-axis remains separated at the dystrophin–actin junction (Fig. 3).

It was once reported that β-dystroglycan is absent in Fukuyama type congenital muscular dystrophy (FCMD), based on immunohistochemical data (26). However, this was later shown not to be the case (27). Furthermore, the locus of the responsible gene of FCMD has been confined to 9q31–33 (28,29) which is different from the dystroglycan gene locus (3p21). A moderate decrease in laminin 2 was reported in FCMD (30).

Lesions of basal lamina. Congenital muscular dystrophy (CMD) is a collective name of muscular dystrophies probably due to defects in various genes. Among them, there is a group in which the α2 subunit (also called merosin) of laminin is absent with an increased expression of the α1 subunit in skeletal muscle basal lamina (31) (merosin-negative CMD; Fig. 3). Defects of dystrophin, the dystroglycan complex and the sarcoglycan complex are not observed. The architecture of basal lamina and connection of the dystrophin-axis with laminin may be incomplete. In other words, the protection system may be weakened and the dystrophin-axis may not be fully functional. This defect of merosin seems to be primarily responsible for the muscle necrosis observed in this disease, because the gene locus of the α2 subunit is localized at 6q22–23 (32) and the causative gene locus of merosin-negative CMD is confined to a region 16 cM from 6q2 (33).

In the *dy* mouse, a defect is located in the α2-gene (34) and the α2-subunit is missing (35,36). Although α1-upregulation has not been examined, similar changes may be present in this case to those in the cases of merosin-negative CMD.

Lesions of sarcoglycan complex or sarcoglycanopathy

Severe childhood autosomal recessive muscular dystrophy (SCARMD) is a disease which is symptomatologically and pathologically very similar to DMD (37). This disease was found in Magreb countries in North Africa (38,39), but later

reported to be present in various American (40), Asian (41,42) and European (43) countries. Immunohistochemically, SCARMD was originally defined as adhalin (50DAG) deficiency (38), in which dystrophin and the dystroglycan complex are present at normal levels. It became clear that SCARMD is not a genetically homogeneous disease, but a group of muscular dystrophies with similar phenotypes caused by the damage to different genes (40,44). Linkage analysis revealed that some cases are linked to chromosome 13q12 (45–47) and another to chromosome 17q (5). Other cases are not linked to either of them. As the adhalin gene is located at 17q21 (6), it is probable that the familial cases of SCARMD which show high probability linkage to 17q are due to primary adhalin deficiency. In such a family, point mutations were discovered in the adhalin gene (5). However, whether these mutations are of pathological significance has not been elucidated.

Meanwhile, it became clear that in SCARMD, all components of the sarcoglycan complex, namely, adhalin, A3b and 35DAG, are absent (Fig. 3), although dystrophin and the dystroglycan complex are present at almost normal levels (48). Presumably, when any one of the components of the sarcoglycan complex is absent, the complex may not be formed and loss of entire components may ensue resulting in the SCARMD phenotype developing independently of any mechanism involving the primary gene defect. Therefore SCARMD can be collectively called sarcoglycanopathy. In some cases, however, only A3b is present to some extent at the sarcolemma. A3b might be fixed in the absence of other two.

In some cases of the advanced stage of SCARMD (41,49), the β1 subunit of laminin is replaced by β2 (s-laminin) which is exclusively expressed in the trough of the secondary furrow of the neuromuscular junction in normal muscle. This replacement may not necessarily lead to weakening of the anchor site of the dystrophin-axis, as laminins 3 and 4 ($\alpha 1\beta 2\gamma 1$ and $\alpha 2\ \beta 2\ \gamma 1$, respectively) are present at the mechanically unstable loci of the furrow.

In any case, it is still premature to describe conclusive features of SCARMD both genetically and phenotypically, because the number of the patients examined is still limited. More cases must be examined in detail to increase our understanding of SCARMD (50,51).

The hamster with autosomal recessive muscular dystrophy, which was at first reported as selective loss of adhalin (52), has been proved to have entire defect of the sarcoglycan complex (53). Although the primary gene defect of the dystrophic hamster has not been identified, the dystrophic hamster can be considered as an animal model of SCARMD. However, we must be cautious because the phenotypes of SCARMD and hamster dystrophy are different. Skeletal muscle is predominantly involved in SCARMD, whereas cardiac muscle is predominantly involved in the hamster dystrophy.

Compound lesions

The subsarcolemmal undercoat of DMD muscle is deficient in dystrophin. This is probably because defective dystrophin is digested.

In DMD, dystroglycan complex expression was reported to be drastically reduced to less than 10% of the normal level, as has been reported for other DAPs by Campbell's group (54,55). However, Ozawa's group reported that the dystroglycan complex is reduced but fairly well preserved (56,57). The fact the expression of utrophin, which binds to the dystroglycan complex, is upregulated in DMD and utrophin is fixed at the sarcolemma (58,59), indicates the dystroglycan complex is required for the fixation. Therefore, the dystroglycan complex must be present in DMD muscle in an amount corresponding to that of utrophin. The dystrophin-axis may be lost and to some extent replaced by the utrophin-axis in DMD (Fig. 3). We do not know the difference in the mechanical strength of the dystrophin-axis compared with that of the utrophin-axis. However, it should be noted that the dystrophin-axis is present in muscle cells, which are frequently subjected to strong mechanical stress, whereas the utrophin-axis is present in various cells which are generally not subjected to such stress. Therefore, dystrophin-axis may be stronger than utrophin-axis. In any case, the protection system becomes weaker in DMD than normal.

Sarcoglycan complex expression is greatly reduced in DMD (54,57) (Fig. 3). This may reflect the fact that the utrophin-axis is present in non-muscle cells where the sarcoglycan complex is not expressed (7,8) and may not bind to the sarcoglycan complex so firmly as the dystrophin-axis. The effects of sarcoglycan complex deficiency in the formation of DMD phenotype must be emphasized, as DMD and SCARMD are phenotypically very similar.

DMD is a compound lesion due to disruption of the dystrophin-axis, which is partially replaced by the utrophin-axis, and loss of the sarcoglycan complex.

In BMD, the dystrophin rod is shorter than normal in most cases (resulting from a deletion of the DMD gene) or longer in others (resulting from duplication of the DMD gene), but the dystrophin molecule has intact A, D and S domains (Fig. 3). The presence of the D domain may ensure the binding of dystrophin to the dystroglycan complex (15,16) and that of the A domain, the binding of dystrophin to the actin filament (60–62). Even severely truncated (63,64) and elongated dystrophin may connect the dystroglycan complex and the actin filaments.

In BMD muscle fibers, the dystrophin-axis is present, the amount of dystrophin is sometimes decreased and utrophin expression is upregulated (59). DAPs are present to some extent (65). Thus, the dystrophin-axis is reduced in number, but fairly well preserved. These observations may be compatible with the classical findings that the severity of symptoms in BMD is independent of the size of the dystrophin molecule but dependent on the amount of dystrophin molecules present (66). Reduction of the dystrophin-axis may result in reduction of the sarcoglycan complex. This indicates again that BMD is a compound lesion similar to DMD.

Lesions of syntrophin complex

At present the pathological significance of the syntrophin complex expression is not known. Studies of the deletion of its binding site in patients gave the conflicting results (67,68).

Much more research must be carried out using various techniques to confirm what we have assumed above. However, at present, we consider that defects in the basal lamina and its fixation system, namely the dystrophin axis, and also in the sarcoglycan complex are manifested as muscular dystrophies.

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